THE DEVELOPMENT OF ANDROGEN-INDEPENDENT PROSTATE CANCER

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The normal prostate and early-stage prostate cancers depend on androgens for growth and survival, and androgen ablation therapy causes them to regress. Cancers that are not cured by surgery eventually become androgen independent, rendering anti-androgen therapy ineffective. But how does androgen independence arise? We predict that understanding the pathways that lead to the development of androgen-independent prostate cancer will pave the way to effective therapies for these, at present, untreatable cancers.

ACTIVATING DOMAIN
Region of steroid hormone receptors that enhances target gene transcription.

ZINC FINGER
Protein module in which conserved cysteine or histidine residues coordinate a zinc atom. Some zinc-finger regions bind specific DNA sequences; others are involved in protein–protein interactions.

HEAT-SHOCK PROTEINS (HSP). Molecular chaperones that are induced during cellular stress. They help regulate cellular homeostasis and promote survival.

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Apart from skin cancer, prostate cancer is the most common form of cancer in men and the second leading cause of cancer deaths in men in the United States[1]. Initial treatment is usually prostatectomy or radiation to remove or destroy the cancerous cells that are still confined within the prostate capsule. However, many patients are not cured by this therapy and their cancer recurs, or they are diagnosed after the cancer has spread. Tumour growth is initially androgen dependent. Androgen ablation (BOX 1), the mainstay of therapy for progressive prostate cancer, causes regression of androgen-dependent tumours, as documented by the work of Huggins over 30 years ago[2]. However, many men eventually fail this therapy and die of recurrent androgen-independent prostate cancer (AIPC). AIPC is a lethal form of prostate cancer that progresses and metastasizes. At present, there is no effective therapy for it. There are several pathways by which AIPC can develop. These pathways provide insights into the mechanism of androgen action and schemes by which cancer cells subvert normal growth control and escape attempts to treat the cancer. Understanding the pathways that lead to AIPC is the first step towards developing therapies for this lethal form of prostate cancer.

Mechanism of androgen action

Why do prostate cancer cells normally need androgens to grow and survive? Prostate cancer growth depends on the ratio of cells proliferating to those dying. Androgens are the main regulator of this ratio by both stimulating proliferation and inhibiting apoptosis. So, prostate cancer depends on a crucial level of androgenic stimulation for growth and survival. Androgen ablation (BOX 1) causes cancer regression because without androgen, the rate of cell proliferation is lower and the rate of cell death is increased, leading to extinction of these cells[3].

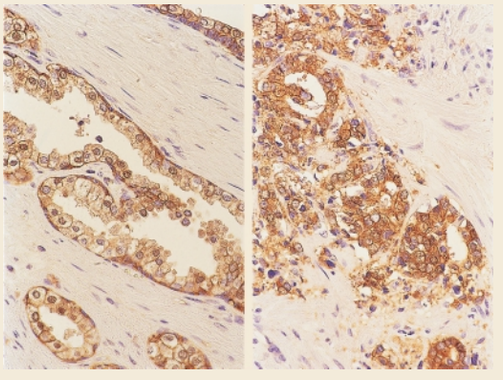
Testosterone — the main circulating androgen — is secreted primarily by the testes, but is also formed by peripheral conversion of adrenal steroids[4]. It circulates in the blood, where it is bound to albumin and sex-hormone-binding globulin (SHBG), with a small fraction dissolved freely in the serum. When free testosterone enters prostate cells (BOX 2), 90% is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (SRD5A2). DHT is the more active hormone, having fivefold higher affinity for the androgen receptor (AR) than does testosterone. The AR is a member of the steroid–thyroid–retinoid nuclear-receptor superfamily[5,6]. It is composed of an amino-terminal ACTIVATING DOMAIN, a carboxy-terminal ligand-binding domain and a DNA-binding domain in the mid-region that contains two ZINC FINGERS. Like other nuclear receptors, in the basal state, the AR is bound to HEAT-SHOCK PROTEINS and other proteins in a conformation that prevents DNA binding. Binding to

Box 1 | **Androgen ablation therapy**

More than 30 years ago, Charles Huggins showed that orchiectomy (removal of the testes) induced the regression of prostate cancer[2]. Since that time, androgen ablation has been the main therapeutic intervention for the treatment of hormone-sensitive prostate cancer[32]. The therapy is very effective in androgen-dependent cancer, but these cancers eventually become androgen independent, and go on to progress and metastasize. Although orchiectomy is an effective means of depleting androgens, pharmacological methods are now available. Gonadotropin-releasing hormone (GnRH) super-agonists (also referred to as luteinizing-hormone(LH)-releasing hormone analogues) downregulate the GnRH receptor in pituitary gonadotropes, leading to the suppression of LH release and inhibition of testosterone secretion from the testis[29]. GnRH antagonists are now in development that immediately antagonize LH release, avoiding the initial stimulation of testosterone secretion that occurs with GnRH super-agonists. Total androgen ablation[31], also referred to as maximal androgen blockade, combines an androgen receptor (AR) antagonist (anti-androgen) with a GnRH inhibitor. AR antagonists also prevent androgens produced by the adrenal glands from binding androgen receptors in the prostate. Total androgen ablation has not yet been shown to prolong survival[33], although it might be helpful in selected patients. It is unclear why the rational use of combination therapy does not improve survival compared with monotherapy, and further study is needed on this important therapeutic question[34]. Use of intermittent androgen ablation is being studied as a means of preventing or delaying the transition of cancer cells to androgen-independent prostate cancer[24], which eventually develops in most cases.

androgens induces a conformational change in the AR that leads to dissociation from the heat-shock proteins and receptor phosphorylation[6], in part mediated by protein kinase A[7]. The ligand-induced conformational change facilitates the formation of AR homodimer complexes that can then bind to ANDROGEN-RESPONSE ELEMENTS (AREs) in the promoter regions of target genes. The activated DNA-bound AR homodimer complex recruits co-regulatory proteins, co-activators or corepressors, to the AR complex. As in other nuclear receptors, the ligand-induced, activated conformation involves a shift in the position of helix 12 of the receptor to form a surface to which co-activators can bind. The co-activators allow interaction of the AR complex with the GENERAL TRANSCRIPTION APPARATUS to stimulate or inhibit target gene transcription[8] (FIG. 1). Many AR target genes have been identified[3], and additional ones are being discovered using cDNA microarray technology[9].

Box 2 | **The prostate**



The main function of the prostate is to produce seminal fluid. The prostate is made up of epithelial glands and a fibromuscular stroma. The glandular epithelium, which gives rise to prostate adenocarcinoma, has three types of cells: basal, luminal secretory and neuroendocrine. There are fewer basal cells and their function is not fully understood, although they secrete components of the basement membrane. A subset of the basal cells might be epithelial stem cells for the luminal epithelial cells[86]. The luminal cells secrete components of prostatic fluid, express the androgen receptor and secrete prostate-specific antigen (PSA) in an androgen-dependent manner. The stroma is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves and some infiltrating cells, such as mast cells and lymphocytes. Some stromal cells are androgen responsive and produce growth factors that act in a paracrine fashion on the epithelial cells. This stromal–epithelial crosstalk is an important regulator of the growth, development and hormonal responses of the prostate[93,94]. The well-organized secretory glandular structure (left) in the normal prostate, accentuated here by immunostaining for E-cadherin, becomes disrupted in invasive prostate cancer (right). (Images courtesy of John McNeal, Stanford University Medical Center, Stanford, California, USA.)

Mechanisms of AIPC development

What triggers the development of AIPC in the first place? Genetic modification is a crucial factor for tumour progression, and the development of AIPC is no exception[10]. But cells have powerful mechanisms that normally guard the genome from mutations. It is possible that, like many other cancers, prostate tumours initially select for genetic changes that increase the likelihood of subsequent mutations[10]. One hint that this process might be important in some prostate cancers comes from research on the phase II detoxification enzyme glutathione S-transferase π. This gene is expressed in normal prostatic epithelium. Here, it catalyses the intracellular detoxification of electrophilic compounds, including some carcinogens, but it is not expressed in more than 90% of prostate cancers owing to methylation of its promoter in a cancer-specific fashion[11]. This is thought to be one of the earliest and most common genomic alterations observed in sporadic prostate cancer. A general increase in the mutation rate would then increase the likelihood of a cell developing ensuing mutations ('multiple hits') that allow the prostate cancer cell to grow independently of androgen[12,13]. Although the necessity of a primary hit is an intriguing possibility, further research is needed to evaluate whether it truly is a prerequisite for the mutations that lead to the development of AIPC.

When in the evolution of advanced prostate cancer do the mutations occur that lead to AIPC? An early study led Cher *et al.*[23] to suggest that "untreated metastatic tumours contain the bulk of chromosomal alterations necessary for recurrence to occur during androgen deprivation", which indicated that mutations might be an early event that is independent of the selective pressure of androgen blockade[23].

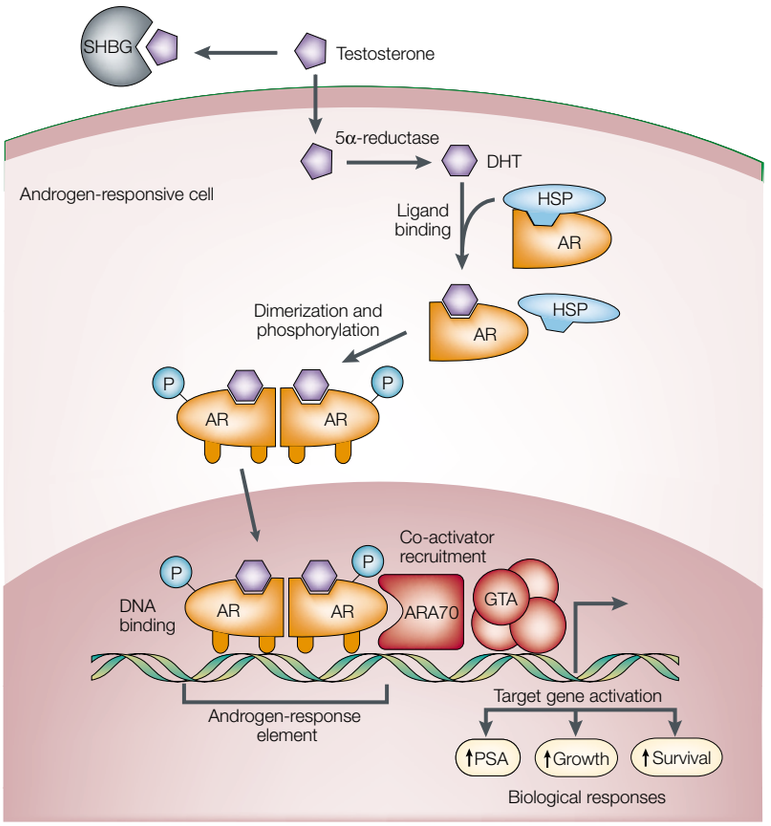


Figure 1 | **Androgen action.** Testosterone circulates in the blood bound to albumin (not shown) and sex-hormone-binding globulin (SHBG), and exchanges with free testosterone. Free testosterone enters prostate cells and is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. Binding of DHT to the androgen receptor (AR) induces dissociation from heat-shock proteins (HSPs) and receptor phosphorylation. The AR dimerizes and can bind to androgen-response elements in the promoter regions of target genes[6]. Co-activators (such as ARA70) and corepressors (not shown) also bind the AR complex, facilitating or preventing, respectively, its interaction with the general transcription apparatus (GTA). Activation (or repression) of target genes leads to biological responses including growth, survival and the production of prostate-specific antigen (PSA). Potential transcription-independent actions of androgens are not shown.

However, many studies have found only a few AR mutations in primary prostate cancer[14]; in comparison, metastatic prostate cancer frequently has mutations in the AR — possibly with a frequency as high as 50% (REFS 14–18). Mutations also might be common in other crucial pathways[10]. Recent investigations therefore support the theory that androgen ablation therapy provides selective pressure to target the androgen signalling pathway[16,18–20]. For example, therapy with the anti-androgen flutamide might select for mutant ARs in which flutamide acts as an agonist rather than an antagonist[18]. Even in the TRAMP (transgenic adenocarcinoma of mouse prostate) model of prostate cancer, in which SV40 large T antigen is overexpressed in the prostate luminal epithelial cells, mutations in the AR frequently develop, and different types of mutation are found in castrated versus intact mice[21,22]. So, the timing of the development of mutations that cause AIPC remains uncertain. Intermittent androgen ablation is considered a possible means of delaying the development of AIPC[24]. If treatment provides selective pressure for mutations that cause AIPC, intermittent treatment might reduce or delay the tendency towards development of mutant cells that become androgen independent. This important issue warrants further study.

The specific types of mutation that lead to AIPC will be discussed in the subsequent sections. We have categorized five potential mechanisms by which AIPC can develop (TABLE 1; FIG. 2). Some of these mechanisms also apply to other forms of steroid-hormone-independent cancer, such as breast cancer (BOX 3).

Type 1: the hypersensitive pathway

One possible mechanism by which a prostate cancer circumvents the effects of androgen ablation therapy is by increasing its sensitivity to very low levels of androgens. Prostate cancers that use this mechanism are not, strictly speaking, androgen independent — their responses still depend on AR and androgen — but they have a lowered threshold for androgens.

AR amplification. There are several potential mechanisms that would allow increased tumour-cell proliferation, despite low circulating androgens in the patient. One mechanism to accomplish this is by increasing the expression of the AR itself. Increased AR abundance leads to enhanced ligand-occupied receptor content, even in the face of reduced androgen concentration. Approximately 30% of tumours that become androgen independent after ablation therapy have amplified the AR gene, resulting in increased AR expression, whereas none of the primary tumours from the same patients before androgen ablation had an AR gene amplification[15,25]. These results indicate that amplification was probably the result of clonal selection of cells that could proliferate, despite very low levels of circulating androgens. Interestingly, patients with tumours that had AR amplification survived longer than patients with tumours that were refractory to ablation therapy but did not have amplification of the AR gene[15]. One possible explanation is that these amplified tumours are more differentiated than other prostate cancers, perhaps allowing the patients to have a better outcome.

Although tumours with AR amplification have increased levels of AR, the signal to proliferate presumably continues to require androgen[15,25]. This is an example of how tumours that seem clinically to be androgen independent could simply have increased their sensitivity to androgens so that they continue to proliferate in a low androgen environment. AR gene amplification that is detected in tumours that are progressing during androgen deprivation monotherapy with gonadotropin-releasing hormone (GnRH) analogues (BOX 1) might be associated with a favourable treatment response to second-line combined total androgen ablation with added anti-androgens[26]. This finding indicates that at least some AR-amplified tumours retain a high degree of dependency on residual androgens that remain in serum after monotherapy[26].

ANDROGEN RESPONSE ELEMENT (ARE). Site composed of hexanucleotide repeats and a spacer, usually in the promoter regions of target genes, that contains the androgen receptor zinc-finger-binding region.

GENERAL TRANSCRIPTION APPARATUS (GTA). A complex of proteins with the potential to facilitate transcription of genes. *In vivo* specificity of gene transcription by the GTA is regulated by interacting transcription factors.

Table 1 | **Mechanisms of development of AIPC**

Type	Pathway	Ligand dependence	AR dependence	Mechanism
1	Hypersensitive AR	Androgen dependent	AR dependent	Amplified AR Sensitive AR Increased DHT
2	Promiscuous AR	Pseudo-androgens Androgen antagonists Corticosteroids Coregulator mutations	Dependent on a mutant AR in LNCaP cells and AR^{ccr} cells	Widened AR specificity Illicit stimulation by non-androgens 'Flutamide withdrawal' (antagonists acting as agonists)
3	Outlaw AR	Androgen independent Ligand independent	AR dependent	Mutant *PTEN* Amplified *HER-2/neu* Activated PI3K Activated MAPK Mutant coregulators
4	Bypass AR	Androgen independent	AR independent	Parallel or alternative survival pathways: • Overexpression of *BCL2* • Activation of other oncogenes • Inactivation of tumour suppressor genes
5	Lurker cells	Androgen independent	AR independent	Malignant epithelial stem cells

AR, androgen receptor; AR^{ccr}, cortisol and cortisone responsive, AR; DHT, dihydrotestosterone; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.

Increased AR sensitivity. A second hypersensitive mechanism for tumour progression was found in animal models of the transition from androgen-dependent prostate cancer to apparent AIPC[27]. This pathway results in high-level expression of the AR, increased stability, and enhanced nuclear localization of AR in recurrent tumour cells. The tumour cells were also hypersensitive to the growth-promoting effects of DHT: the concentration of DHT required for growth stimulation in these AIPC cells was four orders of magnitude lower than that required for androgen-dependent LNCaP CELLS. These results indicate that the AR is transcriptionally active in some models of recurrent prostate cancer and can increase cell proliferation at the low circulating levels of androgen reported in castrated men[27].

Of course, it is also possible that some tumours that contain increased or amplified AR are not merely susceptible to low circulating androgens, but also have constitutive AR activation as described below (see outlaw receptors). Alternatively, tumours might also have amplified levels of co-activators[28], which could facilitate the induction of AR transactivation either by less active adrenal androgens or by lower levels of androgens.

Increased androgen levels. A third hypersensitive mechanism to circumvent androgen ablation therapy is by increasing the local production of androgens, to compensate for the overall decline in circulating testosterone. Prostate cells could increase the rate of conversion of testosterone to the more potent hormone DHT by increasing 5α-reductase activity. This would facilitate continued AR signalling even with significantly lower levels of serum testosterone. In support of this mechanism is the finding that, after androgen ablation therapy, serum testosterone levels decrease by 95%, but the concentration of DHT in prostate tissue is reduced by only 60% (REF. 29). Also, epidemiological studies have shown that certain ethnic groups who have higher levels of 5α-reductase activity have a higher incidence of prostate cancer[30]. Although the frequency of prostate cancer foci is similar in men from different ethnic groups, the proportion who develop clinically apparent cancer is higher in men of African descent than in Caucasians or men of Asian descent[10]. Men of African descent, who have a particularly high rate of prostate cancer, show the highest incidence of a polymorphism in the gene for 5α-reductase. This polymorphism substitutes a valine at codon 89 with a leucine (V89L), and results in significantly higher 5α-reductase enzyme activity. Men of Asian descent, who are at low risk for prostate cancer, have a low incidence of this polymorphism; men of Central- and South-American descent have both an intermediate incidence of the polymorphism and an intermediate risk[30]. In addition to genetic predisposition, it is also possible that, by selection during therapy, tumour cells either acquire mutations in the gene for 5α-reductase or select for increased expression of the enzyme. However, to our knowledge this has not yet been shown to occur in prostate cancer.

Recognition that patients can fail hormone ablation therapy even when very low serum levels of androgens are achieved led to the hypothesis that peripheral conversion of adrenal steroids to potent androgens could be sufficient to sustain the androgen signal, causing tumour growth and failure of androgen ablation therapy[31]. This hypothesis resulted in clinical trials using total androgen ablation (also called maximum androgen blockade) (BOX 1) to block residual androgen action at the AR[31]. However, so far, this therapy does not seem to provide any survival advantage[32–34].

LNCaP CELLS
A widely studied metastatic prostate cancer cell line that is androgen responsive.

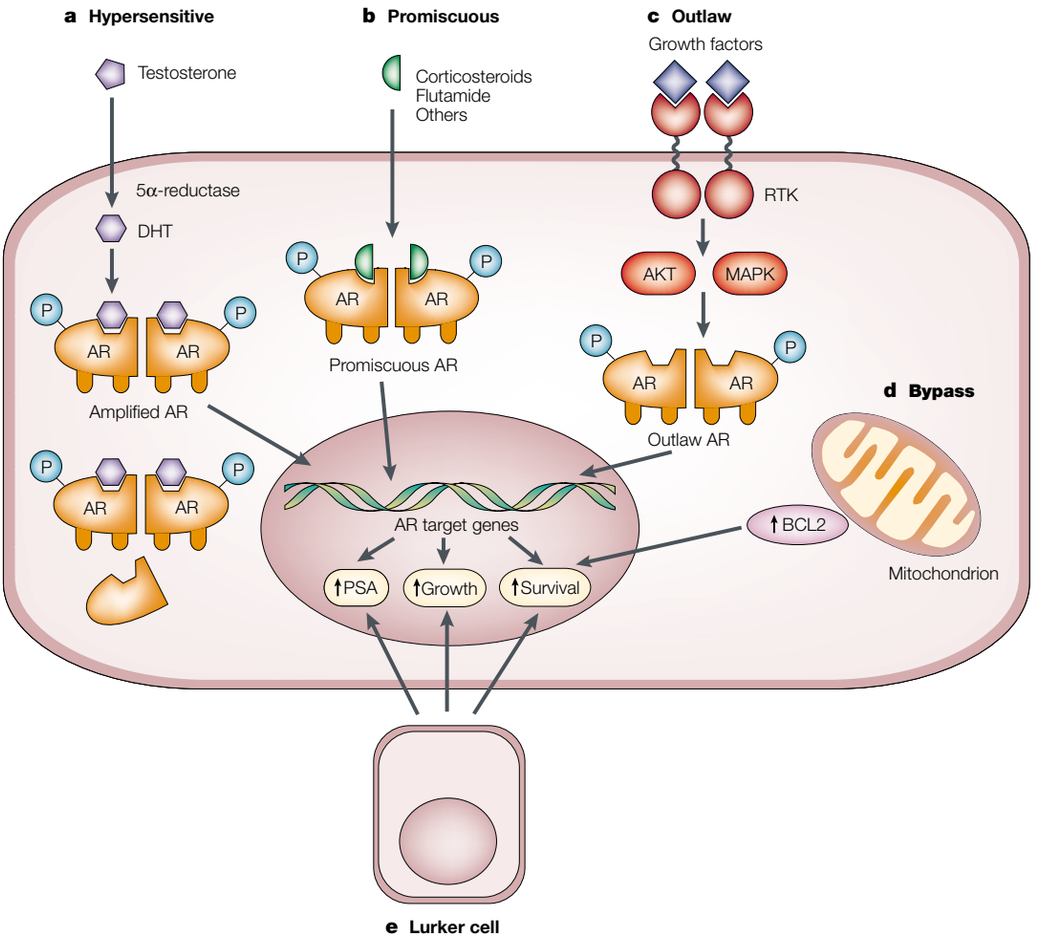


Figure 2 | **Five possible pathways to androgen independence. a** | In the hypersensitive pathway, more androgen receptor (AR) is produced (usually by gene amplification), or AR has enhanced sensitivity (not shown) to compensate for low levels of androgen, or more testosterone is converted to the more potent androgen, dihydrotestosterone (DHT), by 5α-reductase. **b** | In the promiscuous pathway, the specificity of the AR is broadened so that it can be activated by non-androgenic molecules normally present in the circulation. **c** | In the outlaw pathway, receptor tyrosine kinases (RTKs) are activated, and the AR is phosphorylated by either the AKT (protein kinase B) or the mitogen-activated protein kinase (MAPK) pathway, producing a ligand-independent AR. **d** | In the bypass pathway, parallel survival pathways, such as that involving the anti-apoptotic protein BCL2 (B-cell lymphoma 2), obviate the need for AR or its ligand. Finally, **e** | in the lurker cell pathway, androgen-independent cancer cells that are present all the time in the prostate — possibly epithelial stem cells — might be selected for by therapy.

Type 2: the promiscuous pathway

Most AIPCs express the AR protein. Whereas some of these tumours, at least initially, have adapted to the low-androgen environment, others acquire mutations that allow them to circumvent the normal growth regulation by androgens. It seems that many cases of AIPC do not develop from a loss of androgen signalling, but rather from the acquisition of genetic changes that lead to aberrant activation of the androgen signalling axis[21]. These changes are usually missense mutations in the AR gene that decrease the specificity of ligand binding and allow inappropriate activation by various non-androgen steroids and androgen antagonists.

AR mutations. The AR gene is located on the X chromosome and is not necessary for survival, so germ-line loss-of-function mutations in the AR, resulting in the androgen-insensitivity syndrome, are frequent[5]. The incidence of somatic AR mutations within prostate cancer cells is unclear owing to contradictory reports[14]. This is probably due to cellular heterogeneity within tumours, differences in the methodology for detecting mutations, and variations in the stage of tumours examined. Recent results, however, indicate that there is an increased incidence of somatic AR mutations in metastatic samples[14], confirming the earlier data of Taplin *et al.*[16]. Microdissection of tumours and laser capture techniques[35] will probably resolve this controversy, and this method should be considered in all future studies of AR mutations in metastatic specimens. On balance, it seems likely that the frequency of mutations in the AR is significantly increased in tumours after androgen ablation therapy, whereas most studies have reported few AR mutations in primary tumour samples collected before therapy[14,16,17]. This indicates that acquisition of mutations in the AR is likely to be

Box 3 | **Shared features of breast and prostate cancer**

The study of how androgen-independent prostate cancer (AIPC) develops raises interesting basic scientific questions about cancer biology, and there are many parallels with the development of steroid hormone independence in other tumours. The cancer that has provided the most insight into AIPC is breast cancer. Most important is the hormone-dependent nature of these cancers, leading to an interplay between the cancer cell and the endocrine system. In both types of cancer, this crosstalk has resulted in the use of endocrine modulators for therapy. Unfortunately, both cancers can progress to hormone-independent disease. As with prostate cancer cells and androgen receptors, breast cancer cells that express oestrogen receptors are dependent on oestrogens to promote proliferation and inhibit apoptosis. Effective breast cancer therapy in these tumours includes blocking the oestrogen receptor pathway using oestrogen antagonists (anti-oestrogens). The two anti-oestrogens in general use — tamoxifen and raloxifene — both have differential agonist and antagonist activity in various organs and are therefore called selective oestrogen receptor modulators (SERMs)[95]. A subset of patients treated with anti-oestrogens or SERMs will initially respond, but might later recur with oestrogen-independent tumours.

one mechanism for the development of AIPC. It seems reasonable that gain-of-function mutations that lead to a growth advantage by the tumour would be selected for. It is interesting that the loss-of-function mutations in the androgen-insensitivity syndrome are at different positions within the AR than the gain-of-function mutations found in prostate cancer[21].

Although only a few mutations in the AR have been studied in detail, a mechanism for the development of AIPC has emerged from these studies. In cells with these AR mutations, the androgen signal is maintained by broadening the number of ligands that can bind to and activate the receptor. Normally, the AR is specifically activated by testosterone and DHT, but mutations in the ligand-binding domain widen this stringent specificity. As a result, the malignant cells can continue to proliferate and avoid apoptosis by using other circulating steroid hormones as substitute androgens when the level of androgens is low.

The first AR mutation of this type was discovered in LNCaP cells[36]. LNCaP cells express high levels of AR, and androgens stimulate them to grow and express PROSTATE-SPECIFIC ANTIGEN (PSA) — a widely used and clinically important marker for prostate cancer cells. However, owing to a mutation in the AR, other steroid hormones, as well as the androgen antagonist flutamide, activate the AR and stimulate proliferation. Sequencing of the AR gene from LNCaP cells revealed a missense mutation in amino acid 877, which is located in the ligand-binding domain. This mutation results in the substitution of alanine for threonine at position 877 (T877A)[36] (FIG. 3). Molecular studies showed that hormones such as progestins, oestrogens and anti-androgens illicitly bind to this mutant AR and act as agonists[36]. During androgen ablation therapy, it is likely that this mutation undergoes clonal selection, conferring a growth advantage to cells that harbour the mutation[18]. Gaddipati *et al.*[37] examined 24 tumour samples from patients with metastatic prostate cancer and found the T877A mutation in six of the samples (25%), indicating that the mutation is relatively common in patients with AIPC. Promiscuous AR activators include adrenal androgens and metabolic products of DHT[19,38].

PROSTATE-SPECIFIC ANTIGEN (PSA). A serine protease in the kallikrein gene family that is secreted into seminal fluid by prostatic epithelial cells and found in the serum. As it is almost exclusively a product of prostate cells, measurement in blood has proved to be exceptionally useful as a tumour marker for diagnosis of prostate cancer and monitoring the effectiveness of treatment.

The promiscuous receptor mechanism can also explain the clinically observed phenomenon of 'flutamide withdrawal syndrome', in which patients show clinical worsening with flutamide, but then improve when flutamide is withdrawn[39]. Flutamide is an effective antagonist of the wild-type AR and so is used in androgen ablation therapy (BOX 1), but some patients treated with this anti-androgen experience a rapidly rising PSA level. This seems to be due to selection of AR mutations that yield a promiscuous receptor. In a series of bone marrow metastases, T877A mutations were found in 5 out of 16 patients who received combined androgen blockade with flutamide[18]. Cells harbouring these mutant ARs were strongly stimulated to grow by flutamide, whereas patients not treated with flutamide had different mutations that were not stimulated to grow by flutamide. These findings indicate that AR mutations occur in response to strong selective pressure from flutamide treatment[18]. In patients harbouring such tumours, discontinuing flutamide results in initial tumour regression before growth eventually resumes. On a molecular level, the T877A mutation changes the AR response to flutamide from an antagonist to an agonist. Interestingly, the T877A mutant AR does not have the same response to other anti-androgens such as bicalutamide (casodex). Many other mutations in the AR have been identified[40] and are catalogued in the Androgen Receptor Gene Mutations Database. It is unclear how many other mutations use the same promiscuous receptor mechanism and allow prostate cancer cells to become androgen independent.

Crystallographic studies of the ligand-binding domain of the wild-type AR[41] and the T877A mutant AR[42] have recently revealed that substituting alanine for threonine in the ligand-binding pocket explains the ability of the mutant AR to accommodate progesterone and other ligands that the wild-type receptor cannot. Similarly, the CWR22 tumour cell line has an H874Y mutation (substituting tyrosine for histidine) that influences binding of co-activator proteins by affecting the conformation of helix 12 (REF. 43).

The MDA PCa 2a and 2b cell lines, established from a bone metastasis in a patient who had recurrent metastatic disease that developed after orchiectomy[44], also harbour promiscuous ARs[45,46]. Like LNCaP cells, MDA PCa 2a and 2b cells express the AR, and androgen stimulates PSA expression and cell growth. However, the AR has reduced affinity for androgens, and MDA cells are less sensitive to androgens than LNCaP cells[46]. We identified two distinct missense mutations in the AR ligand-binding domain[46]. Double mutations in the AR have been reported previously[40], but never both in the ligand-binding domain. This mutated AR had the T877A mutation, as well as a previously identified[47] leucine-to-histidine substitution at amino acid 701 (L701H)[46]. We reasoned that these two mutations in the ligand-binding domain were likely to change the specificity of ligand binding to the AR (FIG. 3). In fact, the L701H mutation alone decreases the ability of AR to bind and respond to DHT[45]. However, the L701H

mutation also enhances the binding of other adrenal corticosteroids, particularly the glucocorticoids cortisol and cortisone. The T877A mutation has a synergistic effect by increasing the affinity of the AR for glucocorticoids by 300% more than the L701H mutation alone[45]. In this doubly mutated AR, cortisol and cortisone function as AR agonists (hence this mutant is named AR^{ccr}, for cortisol and cortisone responsive), and illicit binding leads to the induction of *AR*-responsive genes such as PSA (FIG. 3). So, in cells with the AR^{ccr} mutation, glucocorticoids can substitute for androgens and promote androgen-independent growth[45]. Because of the high affinity of glucocorticoids for the AR^{ccr}, it is likely that physiological levels of circulating cortisol and cortisone would be sufficient to promote tumour growth in patients with this double mutation. The frequency of this mutation in prostate cancer patients is unknown but the L701H mutation, which is sufficient to render the AR responsive to corticosteroids, has been reported three times[45]. Obviously, this type of patient should not be treated with hydrocortisone. However, some synthetic glucocorticoids have low affinity for the AR^{ccr} and might be useful therapeutically to suppress endogenous corticosteroids[45]. This hypothesis will require further investigation.

Recently, spontaneously occurring AR mutations have been found in the TRAMP transgenic model of prostate cancer[21,22,48]. These differ depending on whether the mice have been castrated. The mutations cluster in three regions of the AR: the highly conserved signature loop in all nuclear receptors; the region flanking the site where p160 CO-ACTIVATORS bind; and the boundary between the hinge and the ligand-binding domain. Consistent with the AR mutations described above, many of the AR mutations found in clinical cancer cases result in decreased specificity of ligand binding and inappropriate receptor activation by non-androgens, yielding a promiscuous AR phenotype[21]. But not all AIPCs with apparently promiscuous ARs harbour mutations in the AR. This has been shown by selecting for AIPC cells in castrated immunodeficient mice[49]. In another example, the LNCaP cell line was continuously selected for by growth in androgen-depleted medium over many passages, and a variant cell line emerged[50]. This cell line — called LNCaP-abl — had a fourfold higher expression of AR and a 30-fold increase in basal AR transcriptional activity compared with the parental LNCaP line. In the LNCaP-abl cell line, casodex — which functions as an AR antagonist in the parental cell line — functions as an agonist[50]. But despite these changes, the AR in LNCaP-abl cells was not amplified and had only the parental LNCaP T877A mutation. Clearly, a mechanism other than mutations in the AR is promoting tumour progression. In these examples of experimental selection for AIPC cells, both in castrated mice and in cultured cells, one possibility is that co-regulatory molecules that interact with and either enhance or repress the AR signal might be responsible for the increase in AR responsiveness[50].

p160 CO-ACTIVATORS
p160 co-activators are a family of ~ 160-kDa proteins that act as co-activators of nuclear receptors. SRC1 and TIF2 are members of this family.

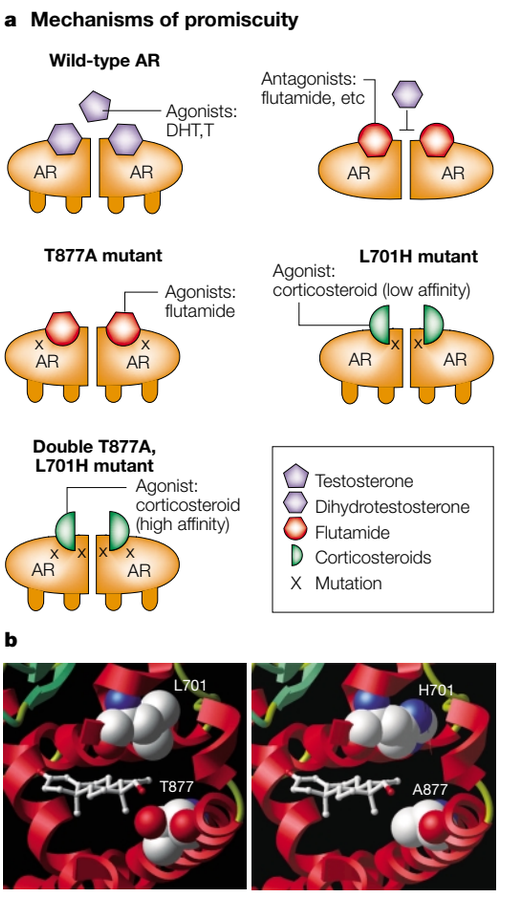


Figure 3 | **The promiscuous androgen receptor.** **a** | Mutations that broaden the specificity of the androgen receptor (AR): in the wild-type receptor, testosterone (T) and dihydrotestosterone (DHT) are agonists, whereas flutamide is an antagonist. The T877A mutant is activated by various non-androgenic steroid hormones, and flutamide also behaves as an agonist. The L701H mutant has reduced affinity for DHT and binds corticosteroids, but when the T877A and L701H mutations are combined, the resulting receptor (AR^{ccr}) has high affinity for corticosteroids. **b** | Models of the ligand-binding sites from the wild-type AR (left) and AR^{ccr} (right) with DHT bound, showing the extra space generated by the mutation of residues 701 and 887. Residues 701 and 877 are shown as space-filling models (carbon, white; nitrogen, blue; oxygen, red), and DHT is shown as a ball-and-stick model. A hydrogen bond can form between DHT and T877, but not between DHT and A877. (Images courtesy of Stanley R. Krystek and John Sack, Bristol-Myers Squibb, Princeton, New Jersey, USA.)

Co-regulator alterations. Several proteins act together with steroid hormone receptors as co-activators and corepressors of transcription[8]. A recent report of a case of androgen-insensitivity syndrome implicated an abnormal co-activator as the defect in androgen action, as the AR did not have a mutation[51]. Modulation of these co-regulatory proteins and their function is likely to be another mechanism by which prostate cancer progresses to AIPC. Breast and ovarian tumours — which can also progress from steroid hormone dependence to independence (BOX 3) — can use this mechanism. For

example, a member of the steroid receptor co-activator 1 (SRC1) family of nuclear receptor co-activators, AIB1, is amplified in some breast and ovarian tumours[52]. This protein interacts with the oestrogen receptor (ER) and enhances the transcription of oestrogen-regulated genes[52]. SRC1 family members seem to function in a relatively large number of tissue types, whereas a co-activator, ARA70, is said to be specific for androgen-responsive genes[53], although divergent results have been reported[54,55]. In the DU145 metastatic prostate cancer cell line, cotransfection of AR and ARA70 specifically enhanced transcription of androgen-responsive genes[56]. ARA70 also facilitates the conversion of several androgen antagonists to agonists in this cell line[56].

Gregory *et al.* recently showed that overexpression of two co-activators, TIF2 and SRC1, occurs in some specimens from recurrent prostate cancers and from prostate cancer cell lines. When combined with promiscuous ARs that have ligand-binding domain mutations, these changes are associated with increased AR activation, even at physiological concentrations of adrenal androgens[28]. The authors believe that most recurrent prostate cancers overexpress co-activators, thereby facilitating AR transactivation and enhancing responses to low levels of androgens. This would represent a combination of the hypersensitive pathway and the promiscuous pathway, and emphasizes the fact that several mechanisms can contribute to a single case of AIPC.

Although overexpression of co-activators is a possible mechanism for creating or enhancing promiscuous ARs in some tumours, a decrease in corepressor expression is equally likely to have similar effects. Again, research on breast cancer is a good model for this mechanism. Decreased expression of the nuclear receptor co-repressor (N-CoR) correlates with resistance to tamoxifen (BOX 3) in patients with breast cancer[57]. Normally, transactivation by the ER is blocked by tamoxifen if N-CoR is bound to the ER. Without the corepressor function of N-CoR, tamoxifen becomes an agonist, leading to activation of oestrogen-responsive genes[57]. Although there are, at present, no reports of similar events in prostate cancer, it is feasible that loss or decrease of AR corepressors would create a promiscuous AR by allowing molecules that normally do not activate the AR to take on the function of agonists. In the case of an androgen-ablated patient, corepressor loss might activate the AR signal for proliferation in the tumour cells, causing AIPC. Conformational changes in the AR, induced by various interacting proteins, are probably crucial for regulation of these events. Determining the crystal structure of these proteins will be vital for increasing our understanding of this mechanism of hormone-independent growth, as well as for developing effective treatment modalities.

Type 3: the outlaw pathway

Steroid hormone receptors that are activated by ligand-independent mechanisms have been referred to as 'outlaw' receptors[58]. An outlaw ER has been described in breast cancer, from which ERs with mutations that are capable of either dominant-positive or DOMINANT-NEGATIVE transactivation of oestrogen response elements were identified[58]. So far, no mutations in the AR have been reported to acquire this type of activity; however, other pathways can subvert the AR into becoming an outlaw.

Growth-factor-activated outlaw pathways. Certain growth factors, such as insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF), can activate the AR, creating an outlaw receptor, and can therefore induce AR target genes in the absence of androgen[59]. IGF-1, the most potent of the factors tested, induced a fivefold rise in PSA secretion in LNCaP cells[59]. These growth factors are ligands for receptor tyrosine kinases and initiate complex intracellular signalling cascades. It is unclear, at present, whether their effect on the AR pathway is direct or is the result of a downstream molecule that is induced in the signalling pathway. An intriguing supportive finding is the discovery that these growth factors seem to be overexpressed in some prostate cancers. Significantly, the AR antagonist casodex completely blocks activation of the AR by IGF-1, KGF and EGF[59]. This indicates that the AR ligand-binding domain is necessary for this outlaw activation. Furthermore, the ability of casodex to block IGF-induced AR activation makes this mechanism an unlikely explanation for AIPC in patients who fail casodex therapy. It is possible that upregulation of growth factor expression — combined with AR mutations — could result in AIPC in such patients, but further research is needed to test this hypothesis. Nevertheless, these experiments[59] had the important impact of highlighting the significance of tyrosine kinases in AR signalling and prostate cancer.

Receptor-tyrosine-kinase-activated outlaw pathways. Studies in breast and ovarian cancers have provided evidence of a connection between nuclear receptor signalling and receptor tyrosine kinases. HER-2/neu (also known as ERBB2) — a member of the EGF-receptor family of receptor tyrosine kinases — is overexpressed in 20–30% of breast and ovarian cancers[60]. HER-2/neu has intrinsic tyrosine-kinase activity and can activate the ER in the absence of oestrogenic ligand. Therefore, overexpression of *HER-2/neu* could lead to oestrogen-independent stimulation of ER-mediated signal transduction pathways. Interestingly, in breast cancer, overexpression of *HER-2/neu* correlates with oestrogen independence[61], probably because HER-2/neu activation indirectly leads to phosphorylation and activation of the ER in the absence of oestrogen[62]. Phosphorylation therefore creates an outlaw ER, resulting in the oestrogen-independent growth of breast cancer cells[62].

The AR can be turned into an outlaw receptor by the same mechanism: *HER-2/neu* is consistently overexpressed in AIPC-cell sublines that are generated from XENOGRAFTS implanted in castrated mice[63], and androgen-dependent cell lines can be converted to androgen-independent cells by overexpressing *HER-2/neu*. Overexpression of *HER-2/neu* can activate AR-dependent genes in the absence of AR ligand[63,64], but not in the absence of AR. However, unlike the effect of IGF-1, the outlaw AR created by *HER-2/neu* overexpression could

DOMINANT NEGATIVE
A protein with an inhibitory signal that overrides or blocks a positive signal for transcription.

XENOGRAFT
A graft of tissue or cells transplanted between animals of different species.

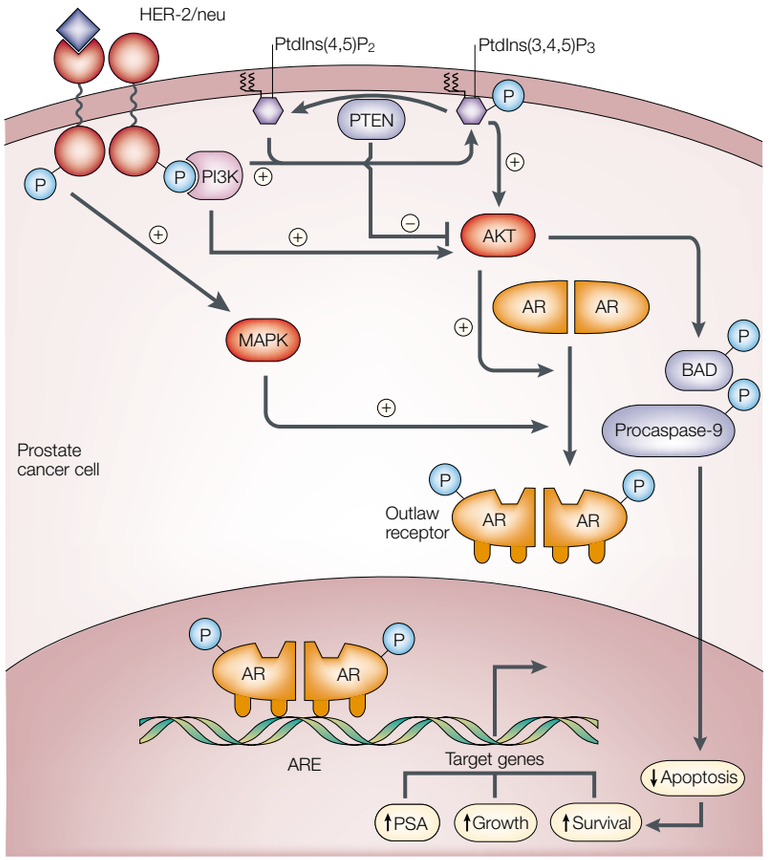


Figure 4 | **How growth factor signal transduction creates outlaw receptors.** In the tumour cells of a patient receiving androgen ablation therapy, HER-2/neu (and possibly other receptor tyrosine kinases) can become overexpressed. HER-2/neu indirectly activates mitogen-activated protein kinase (MAPK). MAPK might phosphorylate the androgen receptor (AR), creating an androgen-independent 'outlaw' receptor. An alternative means by which HER-2/neu (or other pathways) might activate the AR is by activating the AKT (protein kinase B) pathway. In this pathway, activation of receptor tyrosine kinases, such as HER-2/neu, increase the level of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) by activating phosphatidylinositol 3-kinase (PI3K). Another pathway might involve inactivation of the lipid phosphatase PTEN, so that PtdIns(3,4,5)P_3 can no longer be converted back to its substrate, PtdIns(4,5)P_2. AKT is activated by PtdIns(3,4,5)P_3, and might be able to produce an outlaw AR by phosphorylating it. AKT can also activate parallel survival pathways by phosphorylating and inactivating pro-apoptotic molecules such as BAD and procaspase-9. ARE, androgen response element; PSA, prostate-specific antigen.

not be blocked by casodex, indicating that this pathway is independent of the AR ligand-binding domain[63].

Taken together, these findings indicate that activation of HER-2/neu is an important mechanism for the progression to hormone-refractory disease in some breast and prostate cancers. This led to the therapeutic strategy of trying to prevent outlaw receptor formation by blocking the HER-2/neu receptor. Trastuzumab (Herceptin) — a monoclonal antibody against HER-2/neu — was developed as a therapeutic agent to block this pathway[65]. In patients with metastatic breast cancers that overexpress *HER-2/neu*, Herceptin increases the clinical benefit of first-line chemotherapeutic agents[65]. It also shows a benefit as a first-line agent in some patients who have failed other therapies[66]. Might Herceptin be of benefit in patients with AIPC? When tested in androgen-dependent (CWR22 and LNCaP) and androgen-independent (CWR22R) prostate cancer xenografts, Herceptin showed some antiproliferative activity in the androgen-dependent models but, when combined with the chemotherapy drug paclitaxel, it showed additive activity in both androgen-dependent and androgen-independent model systems[67].

Recent research has begun to reveal further details of the HER-2/neu signalling cascade in prostate cancer cells. Yeh *et al.* and colleagues[64] indicate that HER-2/neu could activate the AR through a mitogen-activated protein kinase (MAPK) pathway: inhibitors of MAPK decreased HER-2/neu-mediated activation of the AR. MAPK can phosphorylate the AR *in vitro*, and leads to AR activation in cell lines[64]. From these results, a hypothetical pathway for the development of AIPC can be predicted (FIG. 4). Although there is strong experimental evidence for this mechanism, future investigation is needed to ascertain whether this is truly a pathogenic pathway active in patients who develop AIPC.

The AKT pathway. Direct analysis of cancer samples has led to additional advances in our understanding of AIPC. An example of this was the discovery of the tumour suppressor gene *PTEN*, which was identified as a hot spot for mutations in glioblastoma, breast and prostate cancers[68], and is frequently, functionally inactivated in advanced metastatic prostate cancer[69]. PTEN is a lipid phosphatase that removes the 3-phosphate from 3-phosphorylated inositol lipids, such as phosphatidylinositol (3,4,5)-trisphosphate[70]. 3-phosphorylated inositol lipids are second messengers that activate a protein kinase called AKT or protein kinase B (PKB)[71–73] (FIG. 4). The AKT pathway has been suspected of contributing to tumorigenesis because of its anti-apoptotic activity. AKT phosphorylates and inactivates several proapoptotic proteins, including BAD and procaspase-9 (REF. 74) (FIG. 4). So, in normal cells, by blocking the AKT pathway, PTEN allows cells to undergo apoptosis, whereas tumour cells that have lost PTEN function have increased AKT activity that blocks this signal for apoptosis. AKT has also been shown to regulate cell-cycle progression through a pathway that ultimately downregulates the cell cycle inhibitor p27 (REF. 75).

Might the AKT pathway be involved in prostate tumour progression and the development of AIPC? To test this hypothesis, Graff and colleagues[76] established androgen-independent cell lines (LNAI) from xenografts of LNCaP cells that grew in castrated mice. They found increased AKT activity in the androgen-independent LNAI cell line compared with the parental androgen-dependent LNCaP cells. They also found that overexpressing AKT in LNCaP xenograft tumours accelerated tumour growth and downregulated the expression of p27 in these cells[76]. However, the aetiological role of AKT remains to be confirmed.

AKT might also be an alternative way by which HER-2/neu leads to outlaw AR activation[77,78] (FIG. 4), as HER-2/neu can activate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway[78]. AKT that has been activated by HER-2/neu signalling phosphorylates the

AR at serine (Ser) 213 and Ser791 (REF. 77), turning it into an androgen-independent outlaw receptor. Furthermore, this HER-2/neu-mediated activation of the AR could be blocked by expressing a dominant-negative AKT[77]. The relationship between these results and the activation of AR by the MAPK pathway are at present unclear, and whether this pathway is involved in AIPC development remains to be determined. However, *HER-2/neu* expression seems to increase with progression to AIPC[79], so no matter which of these kinases is responsible for the effect, therapeutic targeting of HER-2/neu in some cases of prostate cancer might be warranted. Recent investigation indicates that the AKT pathway might also be important in the development of tamoxifen-resistant breast cancer[80].

Type 4: the bypass pathway

The mechanisms discussed so far require the presence of the AR and its signalling cascade for the development of AIPC. However, it is also possible that complementary or alternative pathways can be invoked that are capable of bypassing the AR completely. As previously discussed, AR activation stimulates androgen-dependent cancer cells to proliferate, and depletion of androgens results in apoptosis. An effective bypass of the androgen signalling cascade would facilitate proliferation and inhibit apoptosis, even in the absence of androgens and AR. When crucial survival pathways are targeted by therapy, there might be selection for mutations that upregulate parallel pathways that can provide a substitute survival signal. In the case of prostate cancer patients being treated with androgen ablation, blocking the apoptosis signal would be one such pathway for tumour cell survival.

The *BCL2* gene is an obvious bypass candidate gene that can block apoptosis. *BCL2* is not normally expressed in the secretory epithelial cells of the prostate[81]. But *BCL2* is frequently expressed in pre-malignant PROSTATIC INTRAEPITHELIAL NEOPLASIA (PIN), as well as in AIPC[82]. Furthermore, Liu *et al.*[83] detected the emergence of *BCL2* expression in tumours that initially did not express it, by selecting for growth of prostate cancer xenografts in castrated mice. Blocking BCL2 with antisense oligonucleotides delayed the emergence of AIPC in a LNCaP xenograft model[84]. Upregulation of BCL2, then, could bypass the signal for apoptosis that is normally generated by androgen ablation. In support of this mechanism, many cases of AIPC, both in humans and in rodent models, have been found to overexpress *BCL2* (REFS 82,85). However, overexpression of *BCL2* is not essential for the formation of AIPC[85] — presumably because other bypass pathways or one of the other four mechanisms (TABLE 1) can substitute.

Further studies are needed to understand the exact mechanism by which these bypass pathways interact with AR signalling. It remains possible that the pathways directly intersect at a junction yet to be elucidated. Many other oncogenes and tumour suppressor genes, in addition to *BCL2*, could have a similar bypass role in the development of AIPC[10], but discussing each of these genes is beyond the scope of this review. It seems likely that androgen ablation therapy would provide the selective pressure needed for some tumours to adapt to and escape from the effect of therapy by invoking any of these bypass mechanisms.

Type 5: the lurker cell pathway

Androgen ablation fails because cells that are not dependent on androgen for growth take over and the tumour grows in an androgen-independent fashion. As this review has highlighted, there could be several mechanisms by which a cell can become androgen independent and so lead to failure of androgen ablation therapy. However, John Isaacs has postulated[86] that androgen ablation therapy might fail, and AIPC eventually develop, because a subpopulation of androgen-independent tumour cells was present even before therapy was initiated. The putative epithelial stem cells among the basal cells of the prostate are believed to be androgen independent: their rates of proliferation and death are not affected by androgen ablation[3]. According to this model[86], if the epithelial stem cell transformed and became the origin of a prostate cancer, the following events would occur: first, in the presence of androgens, most of the epithelial stem cell progeny would differentiate into androgen-dependent epithelial cancer cells that would comprise most of the tumour; second, after androgen ablation, the androgen-dependent cells would be eliminated but the androgen-independent malignant epithelial stem cells, which have been lurking in the background all along, would remain viable; and third, these malignant epithelial stem cells would continue to proliferate and ultimately result in the relapse of the disease as AIPC. It is tantalizing to consider that prostate tumours resist apoptosis and proliferate by adopting features of normal prostatic stem/progenitor cells, and that basal cells — the putative stem/progenitor cells of the prostate — are androgen independent, just like most advanced prostate cancers[87]. Craft *et al.*[20] provide evidence to support this hypothesis. They showed that the latter stage of androgen independence results from clonal expansion of androgen-independent cells that are present at a frequency of about 1 per 10^5–10^6 androgen-dependent cells. They conclude that prostate cancers contain heterogeneous mixtures of cells that vary in their dependence on androgen for growth and survival, and that treatment with anti-androgen therapy provides selective pressure that alters the relative frequency of these cells, thereby leading to outgrowths of androgen-independent cancers.

This hypothesis draws parallels with certain types of human leukaemia that relapse, despite effective therapy that had reduced the malignant cells to undetectable levels. This might occur because stem cells that are resistant to chemotherapy, lurking in the bone marrow, regenerate the malignant population[88,89]. The potential for a transformed prostate epithelial stem cell to produce androgen-dependent progeny needs further investigation.

PROSTATIC INTRAEPITHELIAL NEOPLASIA (PIN). Dysplastic cellular changes confined to the prostatic epithelium and considered to be a precursor to adenocarcinoma of the prostate.

Concluding remarks

The study of the pathways by which AIPC develops has led to a fascinating overlap between the fields of endocrinology and oncology. The pathways show how malignant cells can hijack the endocrine system and develop alternative signalling pathways to subvert therapeutic attempts to control cell growth by androgen ablation. We do not believe that these five mechanisms exhaust the possibilities and, no doubt, further studies will reveal additional pathways. It is also possible, if not likely, that a single cancer uses several mechanisms either initially or in a multistep progression to AIPC. As prostate cancers use various schemes to subvert normal restraint on cell growth, successful therapy will require an individualistic approach based on the type of AIPC present. Effective therapy of AIPC will require that each patient's cancer be analysed so that a specific targeted therapy can be initiated[90]. To be successful, therapeutic measures will need to rescue the cells from the AIPC mechanism and restore normal growth regulation, or at least block the abnormal stimulation driving cell growth. Such approaches are already being developed[91], as exemplified by the use of Herceptin to treat breast and prostate cancers in which HER-2/neu hyperactivity is the cause of hormone independence[65]. By understanding the mechanisms exploited by the cancers, new therapeutic targets are being recognized[92]. We anticipate that fresh diagnostic measures and additional therapeutic options targeted at the specific defect will soon be added to our armamentarium in our efforts to thwart unregulated cancer cell growth.

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Online links

DATABASES

The following terms in this article are linked online to:

CancerNet: http://cancernet.nci.nih.gov/
prostate cancer | breast tumours | ovarian tumours

LocusLink: www.ncbi.nlm.nih.gov/LocusLink/
albumin | sex-hormone-binding globulin | 5α-reductase | androgen receptor | protein kinase A | glutathione S-transferase π | SRC1 | AIB1 | ARA70 | TIF2 | insulin-like growth-factor-1 | keratinocyte growth factor | epidermal growth factor | HER-2/neu | MAPK | *PTEN* | AKT | BAD | procaspase-9 | p27 | PI3K | *BCL2*

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flutamide | casodex | tamoxifen | Herceptin | paclitaxel

FURTHER INFORMATION

Androgen Receptor Gene Mutations Database:
www.mcgill.ca/androgendb