

# Targeted therapy of cancer: new roles for pathologists—prostate cancer

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The clinical dilemma today in the management of prostate cancer (PCA) is to distinguish men who need definitive treatment from men who have indolent disease. As demonstrated most recently by the randomized Scandinavian trial evaluating the benefit of prostatectomy over Watchful Waiting, surgery significantly decreased the risk of death from PCA. However, this same study also suggests that 19 men need to be treated to benefit one man. Given the high prevalence of the disease, the aging of the population, and the potential morbidity of treatment, the ability to distinguish aggressive from indolent forms of PCA is critical. Treatment for advanced PCA begins with androgen ablation, but eventually hormone-refractory (HR) PCA emerges. Novel therapies are in various stages of clinical trials, including kinase inhibitors, antisense oligonucleotides, and inhibitors of heat-shock proteins. The discovery of novel therapeutic approaches is an active area of clinical research. Eliminating HR PCA before it advances is a high priority in the biomarker field. Therefore, the development of molecular signatures of lethal PCA are critical. In addition, the recent discovery that a significant percentage of PCAs harbor a *TMPRSS2-ETS* gene fusion suggests that targeting either the ETS transcription factors or the fusion product may offer a novel approach to therapy. However, in 2007, the mainstay of treatment for advanced PCA remains androgen ablation therapy as originally introduced in the early 1940s.

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## Which men benefit from aggressive treatment?

### Predicting Risk of Adverse Outcome

In the United States, the prevalence of pathological prostate cancer (PCA) is extremely high and increases with age. One in six men will be diagnosed with PCA during their lifetime. PCA is the third leading cause of male cancer-related death, after lung and colorectal cancer<sup>1</sup> and the American Cancer Society estimates that 234 460 American men will be diagnosed with PCA and 27 350 will die in 2006, representing approximately 10% of all cancer deaths in men in the United States. Notwithstanding the sizeable number of deaths, the majority of cases are non-lethal. Thus, the clinical dilemma is that we are over-treating many men diagnosed today in the post-PSA screening era and inadequately treating those with the potential for the most

aggressive form of the disease—metastatic PCA. As demonstrated most recently by the randomized Scandinavian trial evaluating the benefit of prostatectomy over Watchful Waiting, surgery significantly decreased the risk of death from PCA.<sup>2,3</sup> However, this same study also suggests (albeit with relatively short follow-up) that 19 men need to be treated to benefit one man. Given the high prevalence of the disease, the ease of diagnosis, the aging of the population, and the morbidity of treatment, the ability to distinguish aggressive from indolent forms of PCA is critical.

The current methods of stratifying tumors to predict outcome are based on clinical factors. These factors include Gleason grade (a measure of the extent of glandular differentiation), PSA level at diagnosis, clinical stage (the extent of disease burden and spread), rate change in PSA prior to diagnosis, and the percentage of biopsies that contain tumor cells. Nomograms and multifactor staging schemes have been developed, which aid in the prediction of biochemical relapse (rising PSA) after local (surgical or radiotherapeutic) and potentially curative therapy. Although these clinical formulae are helpful, they do not fully predict outcome and importantly are not linked to the most

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meaningful clinical endpoint—PCA-specific death or development of metastatic disease. Predictive models to determine which patient will die of PCA despite therapy are limited.<sup>4,5</sup> Moreover, these nomograms and multifactor staging schemes do not identify those patients who do not require treatment.

The role of the pathologist is critical for determining tumor grade (Gleason grade) and staging of the disease extent. Recognizing the effects of radiation and androgen treatment may be critical in certain clinical settings and may also help to avoid incorrect grading and staging of the disease. Currently, there is a limited role of the pathologist in the evaluation of the response to targeted therapy or even the determination of which patients might benefit most from targeted treatment. For example, although PDGFR and mTOR inhibitors are in clinical trials, it is unclear if we can accurately determine which patients might benefit because of the limitations with assays using formalin-fixed biopsies.<sup>6,7</sup> Therefore, this review will focus on emerging areas of PCA therapy with the anticipations that, as seen in other areas of cancer therapy, pathology input will also be critical in the area of PCA treatment.

Therapeutic options for hormone-refractory (HR) PCA 2007 include antiandrogen withdrawal, antiandrogen administration, adrenal suppressives, corticosteroids, estrogens, external beam radiation therapy, intravenous bone-seeking radioisotopes, bisphosphonates, and experimental therapies.

## Androgen therapy and the histological correlates of androgen ablation

### Androgens and PCA

Hormonal therapy or androgen ablation is mainly used either alone or in combination with other forms of treatment for progressive PCA (ie, local or distant metastatic disease). The use of androgen ablation was initiated by the pioneering studies carried out by Huggins and Hodges in the early 1940s.<sup>8</sup> They showed that androgens influence the development of PCA and furthermore that by withdrawing androgens, either by surgical castration or biochemical castration with oral estrogens (eg, DES), prostate tumors are dramatically reduced. Although prior work had shown the involvement of androgens in PCA, theirs was the first report of PCA responding to androgens in a clinical setting. Androgen ablation can be achieved either surgically or by administration of hormone analogs that block its action. Several LHRH agonists (eg, goserelin, leuprolide, and buserelin) and androgen receptor (AR) antagonists (eg, steroidal, cyproterone acetate and megestrol acetate; and non-steroidal, flutamide, nilutamide, and bicalutamide) have been used for this purpose. However, despite the initial, often dramatic, response to hormone treatment, PCA

tumor cells re-emerge, which are now insensitive or refractory to antiandrogen therapy.

### Androgens Maintain and Stimulate the Prostate Gland

Androgens regulate the growth, differentiation, and maintenance of the prostatic tissue by binding to their cognate receptors. The AR has three main domains: an N-terminal transcriptional activation domain, a C-terminal ligand-binding domain (LBD), and a central DNA-binding domain containing two zinc-finger motifs and a hinge domain. The binding of androgen to its receptor causes a conformational change in the receptor, followed by dimerization and nuclear translocation of the hormone-receptor complex. Once translocated, the ligand-receptor complex binds to specific androgen response elements (AREs) upstream of the target genes along with several coactivator proteins, leading to the transcriptional activation.

In the adult prostatic epithelia, a fine balance between cell proliferation and apoptosis is maintained by testosterone (see review by Litvinov *et al*<sup>9</sup>). The prostate is mainly composed of stromal cells (smooth muscle cells, endothelial cells, and fibroblasts) and the epithelial cells (basal cells, intermediate cells, neuroendocrine cells, and secretory cells). ARs are expressed in the cells of the secretory epithelium and the smooth muscle cells of the stroma, with the intermediate cells expressing very little or no AR. The circulating androgens regulate the proliferation of stroma, whereas the binding of androgens to receptors on the secretory epithelium influences the gene expression pattern alone and not the proliferation. The secretory luminal cells, however, are highly dependent on androgen for survival, whereas the stromal compartment is not.

The circulating testosterone is taken up by the basal and secretory epithelial cells and is converted to a more potent form, dihydrotestosterone (DHT), by the action of 5- $\alpha$ -reductase. The DHT produced by the basal cells acts on the secretory luminal cells to regulate prostate-specific gene expression (PSA, KL2 etc). The growth factors produced by the luminal epithelial cells traverse back across the basement membrane and act on the stromal cells regulating their growth and gene expression. DHT from the basal cells also acts on the stromal cells, leading to the production of peptide growth factors, the andromedins. These growth factors bind to receptors on the basal and secretory epithelial cells to regulate their growth and survival. The survival of the secretory luminal cells is therefore regulated by the paracrine peptide growth factors secreted by the smooth muscle cells of the stroma. (The proliferative components of the epithelial compartment are the basal cells that mature into neuroendocrine or secretory luminal cells, of which the latter are rendered non-proliferative.) Hence, the proliferation and survival of the basal cells is influenced by the

action of growth factors secreted by the stroma, whereas these growth factors regulate only the survival of the luminal cells.

PCA leads to proliferation of the intermediate cells. The paracrine regulation is converted into an autocrine mechanism, where binding of androgen to the receptors on the intermediate cells leads to the production of growth factors that support their proliferation.<sup>10</sup> Surgical or medical androgen ablation results in massive apoptosis of the luminal cells, without affecting the stromal cells. This is believed to be facilitated by the action of TGF- $\beta$ ,<sup>11</sup> which is expressed by the smooth muscle cells of the stroma. The expression of TGF- $\beta$  is normally inhibited by the action of epithelial growth factors. However, following androgen ablation, the inhibition is removed, thereby increasing the levels of TGF- $\beta$ .<sup>12</sup> Another factor thought to play an important role is VEGF, which is an androgen-responsive gene. The loss of expression of VEGF has been proposed to lead to decreased vasculature, resulting in apoptosis of the luminal cells.<sup>13</sup>

Although the initial response to androgen ablation is encouraging, resulting in apoptotic cell death and PCA regression, it is followed by development of aggressive HR disease. This process may vary in the length of time, ranging from a few months to 2 or 3 years. Disease progression and death are the ultimate outcomes. Hence, several studies have been carried out to understand the molecular mechanisms that lead to the development of androgen-refractory PCA in order to come up with effective treatment strategies. To date, no single mechanism has been found to be solely responsible for the onset of hormone-refractory cancer. The three most prevalent mechanistic explanations include the following: (a) amplification of the AR gene leading to an upregulation of AR mRNA, or mutations of the AR that lead to receptor activation by binding of non-canonical ligands or ligand-independent constitutive activation of the receptor; (b) increased ligand-independent signal transduction leading to the activation and nuclear translocation of the AR; and (c) activation of alternate signaling pathways leading to cancer progression.<sup>14</sup> A recent study by Chen *et al*<sup>15</sup> provides strong evidence for an upregulation of the AR mRNA as the chief contributor to the development of HR PCA.

### Amplification of the AR

High levels of AR have been seen to correlate with the recurrence of cancer.<sup>16,17</sup> Amplification of the AR gene was seen in approximately 20–30% of all HR tumors.<sup>18–23</sup> In addition, there was heterogeneity in the amplification seen within tumors.<sup>22</sup> These studies indicated that since amplification is seen in refractory tumors, only the clones having the amplifications would be selected for further growth in a low-androgen environment, and would

accumulate the chromosomal changes with time to establish aggressive disease. Since the AR gene is amplified, it would suggest that the expression levels would also be upregulated, thereby increasing the sensitivity to low levels of circulating androgens. mRNA and protein expression were therefore examined by several groups. mRNA expression was found to be high in tumors with gene amplification.<sup>19,21,24</sup> However, they were also found to be increased in recurrent tumors that did not have AR gene amplification, suggesting that mechanisms other than amplification are responsible for upregulation of the expression of the AR mRNA.<sup>19</sup> The expression of AR protein in tumors with amplification has been reported to be high.<sup>18,25</sup> However, it was also seen that although protein expression was increased in 80% of HR tumors with amplification, tumors without amplification also showed an increase in protein expression<sup>25</sup> as seen in the case of mRNA expression. Amplification has also been correlated to PSA expression and survival. It was seen that amplification positively correlated with increase in PSA in one study<sup>20</sup> but not in the others.<sup>18,19,26</sup> Furthermore, Ford *et al*<sup>18</sup> showed that there was no difference in the survival of the patients with or without amplification. In addition, studies show that amplification is not a common feature of all HR tumors. A recent study demonstrates that the overexpression of AR, determined at the protein level by immunohistochemistry, is associated with worse clinical outcome even for clinically localized tumors, which are hormone naïve.<sup>16</sup> Combined orchiectomy and bicalutamide treatment did not result in amplification.<sup>27</sup> Hence, all these observations further emphasize the involvement of other mechanisms apart from amplification involved in AR upregulation and transition into aggressive cancers.

In addition to amplification, several studies have also shown the presence of mutations in the AR gene to be responsible for transition into HR state. Mutations identified so far have been compiled in a common database.<sup>28</sup> Most mutations have been identified to map in two regions, the LBD and the hinge region. The frequency of the mutations reported vary substantially. Although several mutations have been identified, the mechanisms involved have been elucidated for a few. Mutations have been widely reported in 10–50% of recurring cancers.<sup>29–32</sup> However, the percentage of cases with AR mutations is probably lower and more on the order of <10% when all cases are taken into account. The majority of mutations reported map to the LBD. Mutations in the LBD have been found to broaden ligand specificity.<sup>33</sup> A classical mutation reported by several groups is T877A, which allows binding of antagonists and other non-canonical ligands.<sup>34–36</sup> Mutations also allow activation of AR by antagonists.<sup>37</sup> In a recent study, it was seen that mutations were present in 29% of tumors prior to therapy and was hypothesized to predispose the

tumor to being aggressive.<sup>38</sup> It was seen that the mutations in untreated PCA samples mapped in the boundary between the hinge region and LBD. Similar mutations in the same region have been reported.<sup>29,30,39–41</sup>

The type of treatment given was also shown to influence the kind of mutation seen.<sup>42</sup> Since reports have shown that mutations are present in 20–40% of the advanced PCAs before therapy,<sup>30,31</sup> it could be hypothesized that the mutations were not induced by therapy but were pre-existent. Therefore, certain clones that can grow in the absence or presence of very little androgens are selected for growth during androgen withdrawal and grow aggressively to re-establish the disease. The frequency of incidence of mutations was seen to increase with disease progression.<sup>29–31</sup> It is generally believed that the relatively infrequent mutations cannot account alone for the incidence of HR cancer.<sup>31,43</sup>

### The Role of Alternate Pathways

Ligand-independent activation of AR by various signaling pathways has also been proposed to be a mechanism that leads to AR-regulated gene expression. This ‘outlaw pathway’ was first described by Culig *et al.*<sup>44</sup> They showed that growth factors were able to activate AR in the absence of androgens. AR has been postulated to be phosphorylated and activated by receptor tyrosine kinases (see below). Her2/neu is overexpressed in androgen-independent cell lines and has been shown to increase ligand-independent activation of AR. In addition, the sensitivity to sub-optimal concentrations of androgen is increased, which further increases the activation of AR.<sup>45</sup> This has been shown to occur *in vivo* in a cell-line model where HER2/neu regulates the phosphorylation of AR through a MAP kinase pathway.<sup>46,47</sup> IL-6 can also activate AR in the absence of androgen.<sup>48</sup> IL-6 has been shown to act through Ser/Thr kinase Pim-1 and tyrosine kinase Ekt.<sup>49</sup> PKA activates AR in a ligand-independent manner.<sup>50</sup>

A variety of coactivators are found to be upregulated in PCA.<sup>13</sup> The expression of certain coactivators like SRC1, TIF2,<sup>51</sup> ARA70,<sup>52</sup> cdc25B,<sup>53</sup> nmt55,<sup>54</sup> and Tip60<sup>55</sup> is found to be increased in HR PCA. It is hypothesized that the increase in coactivator expression leads to an increase in AR-regulated gene expression, for instance, ARA70 increases AR expression, stability, and nuclear translocation.<sup>52</sup> Thus, overexpression and recruitment of coactivator proteins to the AR-binding sites may facilitate the progression of HR PCAs.

Apart from these pathways that act through the AR, other independent pathways have been postulated to explain relapse. The antiapoptotic gene bcl2 was found to be overexpressed in androgen-independent PCA.<sup>56,57</sup> Hence, it has been hypothesized that the upregulation of such antiapoptotic proteins

could allow survival of cells following androgen ablation. In addition, a ‘lurker cell pathway’ has been proposed by Isaacs *et al.*,<sup>58</sup> where clonal expansion of pre-existing androgen-independent cells occurs after androgen ablation. However, this pathway remains to be identified.

### AR Upregulation at the Transcriptional Level

Although several mechanisms have been proposed, as discussed, the detailed mechanism by which tumors are rendered hormone-refractory is poorly understood. Chen *et al.*<sup>15</sup> propose that an increase in AR expression is the key mechanism that leads to androgen-refractory PCA. They show that tumors are not ‘androgen independent’ since this upregulation leads to increased sensitivity to low levels of residual androgens, which promotes tumor growth.

The authors compared the gene expression profiles of isogenic hormone sensitive and refractory xenografts. Analysis of microarray data showed the AR gene to be three- to five-fold overexpressed. However, no differences were seen in the expression levels of any other genes tested. This observation is indeed surprising and places the AR as the principal player in conferring androgen-resistant growth. The authors do mention that the hormone-sensitive xenografts were derived from refractory patients. Hence, other model systems could reveal other genes that are differentially expressed. The upregulation of the AR, as seen by microarray analysis, was further confirmed by overexpressing AR in hormone-sensitive cell lines. Overexpression allowed growth of these cells in both androgen-depleted environment and in the presence of the antiandrogen, bicalutamide. Furthermore, when these cell lines were implanted into castrated SCID mice, an early onset of tumor formation was seen compared with the wild-type mice. They then used short-hairpin RNA against the AR and established that increased AR expression is required for tumor establishment in castrated xenograft models. The process of tumor establishment was shown to be ligand-dependent by overexpressing mutants of LBD. The rate of tumor progression was retarded when the LBD was mutated. They also showed that the action of AR is mediated by its binding to DNA and not merely by modulation of cytoplasmic processes. This was shown by disruption of nuclear targeting and by using a mutant of DNA-binding domain. Disruption of coupling to cytoplasmic proteins like Src on the other hand was shown to have no effect on AR action. Hence, the authors suggest a ligand-dependent mass action model where low levels of circulating androgens can bind to the overexpressed receptor, leading to nucleus-mediated alteration of gene expression eventually resulting in tumor progression. They then tried to block the action of androgen using high concentrations of the antagonist bicalutamide. However, it

was seen to function as an agonist of hormone action by stimulating the expression of PSA. The same phenomenon was observed using other antagonists. To determine whether bicalutamide acts as an agonist in the expression of PSA alone or also other androgen-regulated genes, differences in gene expression patterns between AR-overexpressing and wild-type cells lines treated with bicalutamide were examined. This yielded a set of bicalutamide-induced, androgen-sensitive genes that were expressed in the AR cell line. The expression of the same set of genes was also induced in response to low concentrations of synthetic androgen, R1881. This shows that bicalutamide acts as a weak agonist. To investigate the reason for evolution of an agonistic response from well-established antagonists, chromatin immunoprecipitation experiments were carried out to identify the coactivator/co-repressor complexes that were recruited to androgen-regulated promoters upon treatment with the antagonist. It was seen that overexpression of the receptor led to the recruitment of a smaller subset of coactivators in response to antagonists.

This study indeed addresses a wide range of questions regarding the role of AR in the progression of cancer following therapy. The process is shown to be ligand-dependent and therefore the tumors are not strictly 'hormone independent'. Since AR is seen to be overexpressed in all the xenograft models used in this study, the question arises as to why the level of AR increases following androgen ablation. Although amplification has been proposed as a method by which upregulation of AR expression occurs, the frequency of amplification seen is low to explain the observations made in this study. Moreover, overexpression of AR has been reported earlier in the absence of amplification.<sup>19,59</sup> Hence, it would be interesting to investigate the mechanism leading to the increase in AR expression. Another interesting, but intriguing, aspect of the study is the conversion of antagonists to agonists. This phenomenon can be reconciled within the context of mutations that modify ligand specificity. However, the manner by which mere upregulation leads to this effect needs to be investigated. The study has nevertheless contributed toward a better understanding of progression of cancer, following therapy. Cancer recurrence, therefore, seems to be mediated by more than one pathway, where mutations or activation of AR by other signaling pathways discussed earlier in the absence of upregulation could also play a role. The need to find better therapeutic agents is underscored. Potential therapeutic analogs could be designed to either block the interaction of ligands with the receptor or interfere with DNA binding and binding to cofactor proteins. Additionally, if the signaling pathway that leads to the upregulation of AR is identified, analogs could be designed to block this pathway.

A few examples of novel approaches to targeting PCA progression are presented.

## Antisense Oligonucleotide-Targeted Therapy

PCA-targeted therapy has been directed at the AR pathway. However, more general approaches have been used in the therapy of HR PCA. Numerous clinical trials have evaluated the utility of adding chemotherapy following the development of HR PCA. Novel approaches have focused on inhibiting or reactivating genes that are dysregulated in PCA. Antisense oligonucleotide (ASO) therapy was first introduced by Zamecnik and Stephenson<sup>60</sup> when they reported an oligonucleotide complementary to the 3' end of the Rous sarcoma virus. By using a single-stranded ASO (17–22 nucleotides long), one is able to selectively inhibit the expression of the selected gene's mRNA (see recent review by Gleave and Monia<sup>61</sup>).

One ASO directed against the antiapoptotic gene, *bcl2* has been used in preclinical trials for many types of cancer including PCA. G3139 (Genasense, Genta) was given in combination with docetaxel to men with HR PCA. Partial responses were observed in these phase II clinical trials. However, plans for phase III trials were cancelled due to negative results from trials in other cancer types. Improving ASO for PCA-targeted therapies requires modifying the ASO to be less toxic and more stable. Attempts to target genes more specific to PCA progression are also a major focus of development. Clusterin is a cytoprotective chaperon, which is one of the genes that increase in androgen-dependent PCA models. The clusterin ASO (OGX-011; OncoGenex Tech) has been tested in several phase I clinical trials. OGX-011 was well tolerated and was demonstrated to specifically target PCA cells.<sup>62</sup> Phase II trials in HR PCA are nearly completed and will be presented at this year's ASCO meeting in May 2007 (NCIC IND.165-randomized phase II in 80 pts-taxotere ± OGX-011).

HSP27 is an ATP-independent molecular chaperon that is induced during stress and acts to preserve cellular integrity. HSP27 is one gene that increases after androgen withdrawal and may facilitate the transition of tumor cells to the HR state. Clinical trials for HSP27 ASO (OGX-427) will soon begin phase I trials in Spring 2007 (M Gleave, personal communication).

## Development of AR Inhibitors through Understanding AR Biology

Understanding AR signaling is critical for the development of new therapeutic targets. Cross talk between growth factors and AR pathways is an area of significant investigation in PCA. IGF-1, IL-6, and EGF/TGF- $\alpha$  may play a role in PCA development and progression. Recent work by Guo *et al*<sup>63</sup> demonstrates that the AR can be phosphorylated by tyrosine kinase Src. This work suggests that growth factors such as EGF, heregulin, and IL-6 may play a direct role in the activity of the AR. In

addition, their work may explain why the AR can remain active even in states with low androgens, such as during chemical or surgical castration. Understanding this aspect of AR biology might suggest a means of developing kinase inhibitors for the AR to prevent loss of response to antiandrogen therapy.

### Other Drugs in Clinical Trials

Other approaches that are in clinical trials include vaccines and immune stimulants (eg, GM-CSF and anti-CTLA4), angiogenesis inhibitors (eg, bevacizumab or Avastin), and chemotherapeutics (eg, epothilones-anti-microtubular agents). Specific targeted therapy includes inhibitors of mTOR (RAD001) and prostate stem cell antigen monoclonal antibodies. Further therapeutic targets are sonic hedgehog signaling, integrins, PSMA (J591), and HDAC (valproate, PXD-101, and MGC0103).

### Novel Chemical Genomics Approaches to Identifying Regulators of AR Signaling

Perhaps one of the more exciting developments in the field of chemical genomics is the recent proof of concept experiment that identified two inhibitors of the HSP90 as novel agents to regulate AR signaling.<sup>64</sup> This multi-step study design first required identifying a molecular signature associated with androgen repression by using the hormone-sensitive LNCaP PCA cell line and comparing gene expression profiles with and without the presence of androgen. After identification of 27 AR genes associated with the androgen-repressed state,<sup>65</sup> a bead-based multiplex assay (Luminex Beads) was developed to identify which genes from approximately 2500 compounds produced the same expression signature in LNCaP cells as was originally observed by androgen deprivation. Twenty compounds demonstrated this androgen repression signature without being cytotoxic *in vitro*. One compound was rapamycin, the mTOR inhibitor, which is currently being investigated in clinical trials on localized PCA. Two structurally similar compounds, celastrol and gedunin, were also identified. As little was known about the effect of these two plant compounds on AR signaling, LNCaP cells were exposed to varying concentrations of both the compounds, which demonstrated a decrease in AR, PSA, and growth inhibition. The second key to this chemical genomics approach was to try and determine a potential mechanism of action. One significant limitation has been the amount of time required to work up individual compounds. To facilitate this process, Lamb *et al*<sup>66</sup> developed a connectivity map approach to help screen for candidate therapeutic targets. (see Broad Website for introduction to the connectivity map [http://www.broad.mit.edu/genome\\_bio/connectivitymap.html](http://www.broad.mit.edu/genome_bio/connectivitymap.html)). In short, they

performed expression array analysis on three cell lines by exposing them to varying concentrations of over 164 compounds. Many of these compounds are FDA-approved drugs. The idea is that by entering a gene profile into the database, for example, the signature of LNCaP cells after exposure to celastrol or gedunin, one could identify drugs that produce similar effects. By using the connectivity map approach for screening, they determined that celastrol or gedunin produced a similar signature to four HSP90 inhibitors. Interestingly, inhibitors of HSP90 chaperon protein are being used in clinical trials for HR PCA. Additional laboratory studies demonstrated that celastrol inhibits HSP90 by a mechanism different than the current inhibitors, suggesting that this experiment leads to the identification of a plant compound that may add to the effectiveness of currently available inhibitors of HSP90 for the treatment of PCA.

This approach is an exciting proof of principle. It also demonstrates how carefully designed discovery-based studies can lead to hypothesis-based drug development.

### Effects of Common Androgen Ablation Treatment on the Prostate

Antiandrogens such as flutamide or bicalutimide effectively block the effect of androgen on the AR, and LHRH analogs, Lupron and Zoladex, diminish testosterone (T) and DHT profoundly. The morphological changes observed in androgen-treated PCA have been well documented and include loss of glandular architecture, cytoplasmic vacuolization, and nuclear pyknosis as described by Reuter<sup>67</sup> and others. Treated PCA exhibits a paradoxical high Gleason score, but its proliferation rate and degree of aneuploidy is less than those of grade-matched, untreated tumors. It is important to note that the value of the Gleason grading system is its time-honored correlation to outcome in the pretreatment scenario (ie, prior to surgery or radiation). There are no data validating the prognostic value of Gleason grading after therapy, specifically after hormonal therapy. Thus, grading of androgen-treated PCA by the conventional Gleason system may be misleading and should be avoided.<sup>67,68</sup>

Finasteride is a drug used by millions of men to treat the symptoms associated with benign prostatic hyperplasia (BPH). Finasteride is an inhibitor of one of the two subtypes of the enzyme 5- $\alpha$ -reductase (SRDA2), which blocks the conversion of T to DHT, and was recently shown to prevent or delay the detection of PCA as part of the Prostate Cancer Prevention Trial.<sup>69</sup> Men treated with finasteride have a modest increase in serum T levels and intraprostatic DHT levels are diminished by as much as 80%. All in all, this would suggest that treatment-related pathological alterations should be

less dramatic in men treated with finasteride than with antiandrogens or LHRH analogs.

The effects of finasteride on the prostate gland have been best characterized in the prostate tissues of men treated for lower urinary tract symptoms associated with BPH. In that setting, the prostate gland is known to shrink in size due to atrophy of the benign secretory epithelium and to a lesser extent the prostatic stroma composed predominantly of smooth muscle. Some investigators have discussed a preferential decrease in size of the gland in the transition zone as compared with the peripheral zone where BPH and PCA, respectively, are believed to most commonly arise.<sup>70</sup> Light-microscopic evaluation of the prostate gland treated with finasteride demonstrates variable atrophy of the benign secretory epithelium. Depending on the degree of treatment and patient-to-patient differences, the extent of this atrophy can range from global, that is involving the majority of epithelial cells, to partial, involving some glands but not all in a uniform manner. It is also worth noting that these changes are not specific to finasteride and cannot be differentiated from other causes of atrophy seen in patients without finasteride treatment.<sup>71</sup>

There are limited data on the effect of finasteride on PCA in humans. To date, most studies have concentrated on the effect in animals and tumor cell lines. Yang *et al*<sup>71</sup> reported on a small series of prostate needle biopsies obtained from men treated with finasteride. In this non-randomized, underpowered study, there were no significant differences seen between the treatment and control groups with respect to all of the parameters examined, including atrophy, Gleason grade, amount of tumor identified, or other histological features. The study did find a trend toward higher-grade PCA in the placebo group, however, this was not statistically significant. The authors did express some reservations regarding how sampling was performed using multiple 18-gauge needle biopsies, which may have missed the extent of the finasteride effect on the 39 biopsies from finasteride-treated men.

We recently performed a formal study to examine the morphological alterations of finasteride therapy on PCA and compared these changes to those associated with LHRH agonist.<sup>72</sup> We reviewed 56 PCA cases from patients who were treated with finasteride for at least 6 months as well as control cases (no treatment and LHRH agonist treatment prior to surgery). The pathology review was conducted without the knowledge of the patients' treatment status. Histological hormonal treatment effects included apoptosis, vacuolated cytoplasm, pyknotic nuclei, and small irregular glands. No consistent hormonal therapy effects were observed with finasteride treatment as compared with LHRH agonists. Surprisingly, 'hormonal effect' was also observed in a control group. Therefore, no distinct morphological changes can be attributed to long-term finasteride treatment. These findings are

consistent with a recent study that did not observe alterations in Gleason scores due to finasteride treatment.<sup>73</sup>

## Predictive markers of PCA progression

To date, there are no definitive molecular biomarkers to predict PCA progression. Recent examples of individual biomarkers associated with PCA disease progression include EZH2,<sup>74,75</sup> MTA1,<sup>76</sup> Muc1,<sup>77</sup> and *TMPRSS2-ERG* fusion PCA.<sup>78</sup> Individual biomarkers may not be robust enough in clinical practice given the heterogeneity of PCA. Therefore, molecular signatures or panels of gene might be a better approach to developing prognostic biomarkers. Examples of molecular signatures from Glinsky *et al*<sup>9,80</sup> represent good first efforts. However, most of these studies have used highly selected cases for the development of molecular signatures and used PSA biochemical failure as the study outcome. Future studies should focus on well-defined patient populations with long-term follow-up for the development of clinically useful prognostic biomarkers. A recent example from our group is the evaluation of  $\alpha$ -methylacyl CoA racemase for men with clinically localized cancer using PCA-specific death as the outcome.<sup>81</sup> We also recently developed a 12-gene model<sup>82</sup> building on work that used a combination of proteomic and expression array data.<sup>83</sup> We identified a set of 40 genes with concordant dysregulation that could be evaluated by quantitative immunohistochemistry. Using linear discriminant analysis, we determined that the optimal model to predict PCA progression consisted of 12 proteins. Using a separate patient population, the transcriptional levels of the 12 genes encoding for these proteins predicted PSA failure in 80 men following surgery for clinically localized PCA ( $P=0.0015$ ). In an external validation study (described below), the 12-gene model can distinguish men who die of PCA from those with long-term, disease-free survival. This study demonstrates that cross-platform models can lead to predictive models with the possible advantage of being more robust through this selection process.

## Validation of Molecular Signatures of PCA Progression using the Orebro Watchful Waiting Cohort

Through integration of array data at the transcriptome and proteome, our group identified two molecular signatures of advanced PCA, the 12-gene (described above) and 9-gene models,<sup>82,83</sup> which were significantly associated with PSA-failure after prostatectomy. We tested these two signatures in relation to PCA death in the Orebro Watchful Waiting cohort (Mucci *et al*, submitted). Protein expression of these genes was determined by immunohistochemistry on tissue microarrays. We constructed risk scores based on the molecular and

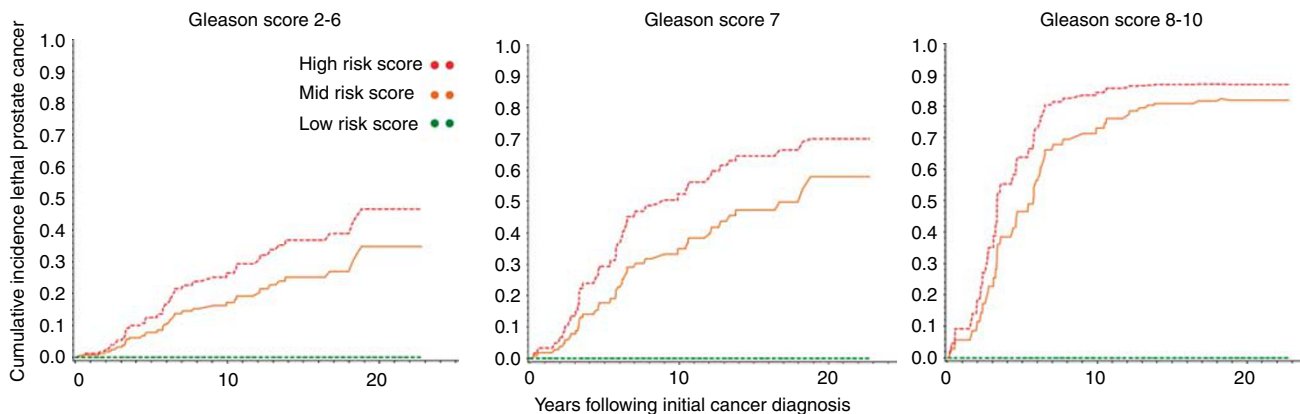
clinical information to predict time to PCA death using Cox models. The 12-gene model classified 17% as high, 75% as intermediate, and 8% as low risk. No cancer deaths occurred in the low risk group. The adjusted cumulative incidence difference was 37% (95% confidence interval (CI) 27–49) in the intermediate and 52% (95% CI 42–62%) in the high risk groups (Figure 1). The 9-gene signature also predicted lethal disease, independent of clinical markers. From 18 unique genes, we identified 5 genes significantly associated with lethal disease. The 5-gene model performed as well or better than the larger gene sets; men in the highest risk quintile using the 5-gene model and clinical parameters had a 58-fold increased risk of progression (65% risk difference) in lethal cancer compared with the lowest risk quintile, with improvement in the concordance index over clinical markers alone. These data demonstrate the potential utility of molecular signatures of PCA death. However, the signatures were not perfect; not all men with the 12-gene signature died of the disease, whereas a proportion of men characterized as low risk by the 9-gene signature developed lethal cancer. Moreover, the majority of deaths occurred in the intermediate risk groups, with mixed discriminatory ability, reflecting the need for better markers to classify outcomes.

### TMPRSS2-ETS Fusion in PCA

By applying a new bioinformatics approach, our group discovered that ERG or ETV1 (both members of the ETS family of transcription factors) were overexpressed in the majority (50–70%) of PCAs and were mutually exclusive across several independent gene expression data sets, suggesting that they may be functionally redundant in PCA development.<sup>84</sup> Because the ETS family of transcription factors has previously been seen in the genomic translocation of

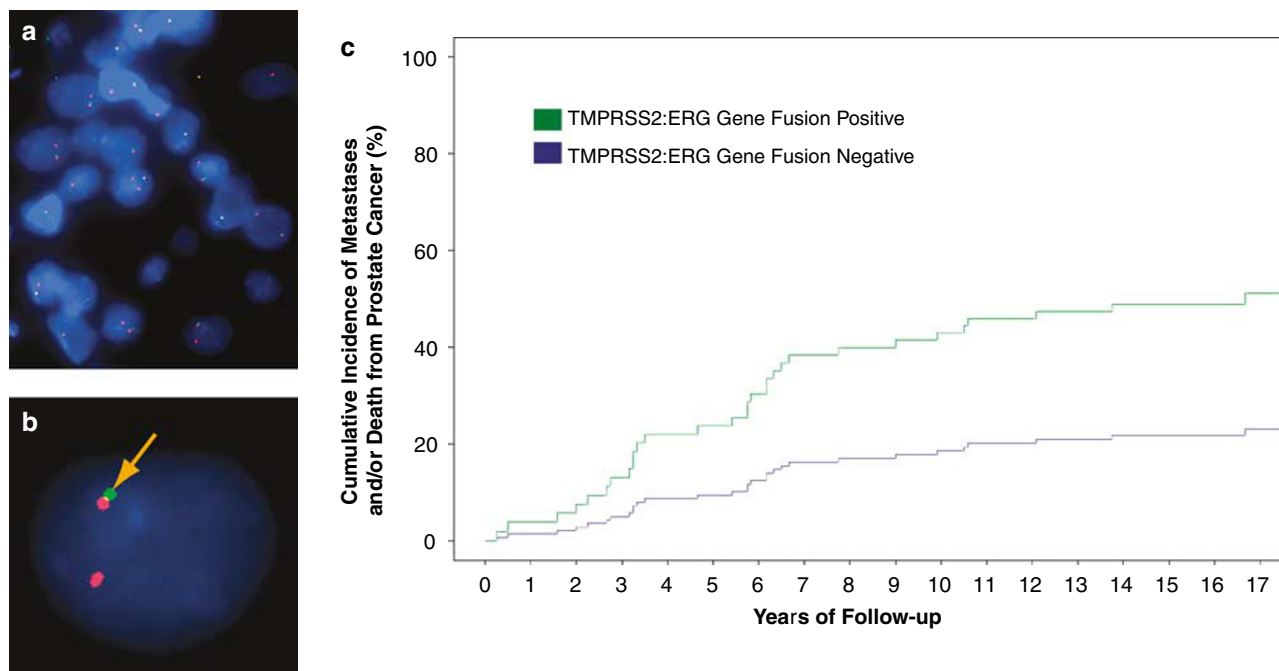
the Ewing's family tumors, AML and other rare tumors, we explored the possibility that they were part of a translocation in PCA. When the ERG cDNA transcript was evaluated exon by exon, we determined that overexpression was typically seen at the distal (3' end) but not the proximal portion (5' end). By sequencing the distal portion of the overexpressed ERG gene, we identified that ERG was fused to another gene, *TMPRSS2*. Whereas hematological malignancies are often characterized by chromosomal rearrangements (ie, translocations), most solid tumors have a plethora of non-specific chromosomal aberrations. Thus, the identification of this fusion between the prostate-specific, strongly androgen-regulated gene *TMPRSS2* (21q22.3) to *ERG* (21q22.2), or *ETV1* (7p21.2) was a surprising discovery. Using other methods to validate these findings (ie, RT-PCR or fluorescence *in situ* hybridization (FISH)) in human PCA samples, we determined that the *TMPRSS2-ETS* fusion is seen in approximately 50–70% of all cases examined. Multiple groups from around the world have confirmed the finding that the *TMPRSS2-ETS* fusion in PCA is a common event.<sup>85–90</sup>

We recently demonstrated that *TMPRSS2-ERG* gene fusion PCAs are significantly associated with PCA specific death or development of metastases<sup>78</sup> (Figure 2). By studying the Örebro Watchful Waiting cohort, we observed 15% (17/111) *TMPRSS2-ERG* fusion in this non-PSA-screened cohort. We identified a statistically significant association between *TMPRSS2-ERG* fusion and PCA-specific death (cumulative incidence ratio = 2.7,  $P < 0.01$ , 95% CI = 1.3–5.8). These data suggest that *TMPRSS2-ERG* fusion PCA may have a more aggressive phenotype, possibly mediated through increased ERG expression. The results also suggest that future studies will have to evaluate whether fusion PCA progresses through alternate molecular pathways, and would also be vulnerable to different drug therapies than non-fusion PCA.



**Figure 1** Validation of molecular signatures of PCA progression using the Örebro Watchful Waiting cohort. The 12-gene model classified 17% as high, 75% as intermediate and 8% as low risk over all. Here, we present cumulative incidence broken down by Gleason score categories. No cancer deaths occurred in the low risk group. The adjusted cumulative incidence difference was 37% (95% CI 27–49) in the intermediate and 52% (95% CI 42–62%) in the high risk groups.





**Figure 2** (a) Using this multicolor FISH probe system, a nucleus without ERG rearrangement demonstrates two pairs of juxtaposed red and green signals. Juxtaposed red-green signals sometimes form a yellow fusion signal (b, arrow). A nucleus with an ERG rearrangement shows replacement of one juxtaposed red-green signal pair with a single red signal for the translocated allele). (c) In a cumulative incidence regression model, we evaluated TMPRSS2-ERG as a determinant for the cumulative incidence or metastases or PCA-specific death. We observed a significant difference in survival in favor of TMPRSS2-ERG gene fusion negative cases. The cumulative incidence ratio was 2.7,  $P < 0.01$ , 95% CI = 1.3–5.8).

In summary, targeted therapy for PCA has focused on modulating AR biology in the setting of HR disease. The effectiveness of novel agents is still unproven despite numerous phase II and III clinical trials. Intense efforts to identify molecular pathways perturbed in subclasses of PCA are underway, including *TMPRSS2-ETS* fusion PCA. The role of the pathologist is not yet as well defined in the field of PCA-targeted therapy, but in the future should parallel their role for other cancers where targeted therapy has been more successful.

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