Molecular pathology of prostate cancer: the key to identifying new biomarkers of disease

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Abstract

Microarray technology has recently accelerated the study of the molecular events involved in prostate cancer, offering the prospect of more precise prognosis and new therapeutic strategies. This review summarises current knowledge of the molecular pathology of prostate cancer. The expression and function of numerous genes have been shown to be altered in prostate cancer. Many of these genes are involved in cell cycle regulation, steroid hormone metabolism or regulation of gene expression. The mechanisms by which androgen independence arises are discussed, including cross-activation, gene amplification and point mutations of the androgen receptor. Analysis of changes in the levels of expression of large numbers of genes during prostate cancer progression have provided a better understanding of the basis of the disease, yielding new molecular markers, such as hepsin, with potential use in diagnosis and prognosis.

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Introduction

Prostate cancer represents one in ten cases of cancer in men, and 200,000 deaths per year, worldwide (Parkin et al. 2001). Because the disease is more common among older men, its incidence is expected to increase as the population ages (Kirby et al. 2001). Prostate tumours, although slower growing than most tumours, vary widely in their aggressiveness (Chodak et al. 1994). The challenges of deciding which patients require immediate treatment, and of developing new therapies, particularly for hormone-refractory tumours, should be eased by a rapidly increasing body of knowledge of the molecular biology of prostate cancer.

Numerous molecular abnormalities have been described, including chromosomal loss or gain (reviewed by Roylance et al. 1997), gene amplification, mutations leading to increases or decreases in gene expression, and mutations leading to changes in function of the protein. The challenge is to use this information to develop better diagnostic and prognostic indicators and to identify new targets for therapeutic intervention. Several recent large-scale studies of gene expression in prostate cancer, using cDNA microarrays, tissue microarrays and other methods, may contribute much towards these goals (Dhanasekaran et al. 2001, Stamey et al. 2001, LaTulippe et al. 2002, Rhodes et al. 2002). The molecular events that occur in the development of androgen independence have also been clarified ( Feldman & Feldman 2001), particularly by a number of reports of ligand-independent cross-activation of the androgen receptor (AR) (Culig et al. 1994, Craft et al. 1999, Sadar 1999, Godoy-Tundidor et al. 2002, Ueda et al. 2002).

Genes involved in prostate cancer development

Not surprisingly, many genes associated with cell cycle regulation and cell proliferation have been implicated. Mutations of p53 are common in prostate cancer, particularly in advanced disease (Meyers et al. 1998). Aberrant nuclear accumulation of p53 protein is a negative prognostic factor for disease-free survival (Leibovich et al. 2000, Quinn et al. 2000). Furthermore, patients with low-level accumulation of p53 had a high probability of worse prognosis if p53-positive nuclei were located near each other (Quinn et al. 2000). p53 and its
downstream effector, p21, have been implicated in the development of androgen independence (see section 'Molecular biology of androgen independence').

The tumour suppressor gene, PTEN, is located on chromosome 10q23 in a region in which loss of heterozygosity frequently occurs in prostate cancer (Cairns et al. 1997), and encodes a phosphatase that negatively regulates cell cycle progression. Loss of PTEN at DNA and protein level has been reported in 25–33% of advanced prostate tumours (Cairns et al. 1997, McMenamin et al. 1999), and at varying frequencies in clinically localised prostate cancer (Cairns et al. 1997, Halvorsen et al. 2003). Loss of PTEN has been associated with increased Gleason score (McMenamin et al. 1999) and risk of clinical recurrence (Halvorsen et al. 2003). The protein kinase Akt is inhibited by PTEN, and a significant downstream result of this is downregulation of the expression of genes that are under the transcriptional control of the androgen receptor (Nan et al. 2003).

The c-met proto-oncogene encodes the hepatocyte growth factor/scatter factor receptor. Its expression increases with Gleason score (Pisters et al. 1995), and more than 90% of metastatic specimens were positive for c-met, in contrast to 50% of the primary samples examined in the same study (Knudsen et al. 2002). Interestingly, Met expression was particularly high in bone metastases and also occurs in normal bone stroma, raising the question of whether it is partly responsible for the tendency of prostate tumours to metastasise to bone sites (Knudsen et al. 2002).

The significant role played by the HER2/neu (c-erbB2) oncogene in breast cancer led to speculation that it may also be important in prostate cancer. However, it is expressed in the latter disease at much lower levels, similar to HER2/neu amplification-negative breast cancer, and in only a minority of patients (Jorda et al. 2002). Although immunohistochemistry failed to detect HER2/neu in 72 samples from locally recurrent or metastatic prostate tumours (Savananinen et al. 2002), an animal model has suggested a possible role for HER2/neu in androgen independence disease (see section 'Molecular biology of androgen independence').

Telomerase reactivation was also detected in 14 of 24 cases of prostate cancer, but not in 12 controls (Meid et al. 2001), and telomerase mRNA levels were greater in malignant than in non-malignant prostate tissue (de Kok et al. 2002). Another study found that E2F4, a transcription factor involved in cell proliferation, was over-expressed at both mRNA and protein levels in malignant radical prostatectomy specimens (Waghray et al. 2001). Other proliferation-related genes have recently been implicated by microarray studies (see below).

Prostate growth is normally regulated by activation of the AR. On binding dihydrotestosterone or similar steroid hormones, the nuclear AR dissociates from heat shock proteins and binds to specific promoters to stimulate transcription. Mutations and alterations in expression of the AR and related proteins are clearly important in prostate cancer.

The possible significance, for prostate cancer development, of two microsatellite repeat polymorphisms (CAG and GGN) in the AR has been investigated. Shorter alleles of the CAG microsatellite repeat polymorphism have been linked to risk of advanced prostate cancer (Ingles et al. 1997) and diagnosis at a younger age (Bratt et al. 1999, Santos et al. 2003), although not to overall risk of prostate cancer (Ingles et al. 1997, Stanford et al. 1997). Conflicting evidence exists as to whether shorter alleles of the GGN repeat confer a greater risk of prostate cancer (Stanford et al. 1997, Correa-Cerro et al. 1999).

The role of the AR in androgen-independent prostate cancer will be discussed in more detail later. In the steroid 5α-reductase gene, which activates testosterone by converting it to dihydrotestosterone, an amino acid substitution conferring greater enzyme activity was associated with a slightly greater risk of prostate cancer (Makridakis et al. 1997). Allele frequencies at a polyA microsatellite in the gene encoding the receptor for vitamin D, another steroid hormone that influences growth of prostate cancer cells (Zhuang & Burnstein 1998), differed between prostate cancer patients and controls (Ingles et al. 1997). Twenty per cent of controls were homozygous for short alleles (<17 repeats), compared with none of 26 patients with advanced disease (Ingles et al. 1997).

A number of genes involved in cell adhesion are involved in the control of prostate cancer growth. E-cadherin was shown to have lower activity in prostate tumours than in normal matched tissue (Rashid et al. 2001). The PC-3 human prostate cancer cell line is deficient in β-catenin, but its restoration reduces the proliferation rate (Ewing et al. 1995). Increased concentrations of the transmembrane glycoprotein KAI1 suppress the development of metastasis in a rat model (Dong et al. 1995).

Glutathione-S-transferase, part of a pathway protecting cells from oxidative damage, shows a strong association between inactivation by hypermethylation and prostate cancer development (Goessl et al. 2001). The oestrogen receptor α is also frequently inactivated by methylation in prostate tumours, but not in benign prostate tissue from the same patients (Sasaki et al. 2002).

Table 1 summarises some of the molecular aberrations observed to date in prostate cancer.
Molecular biology of androgen independence

Many prostate tumours, although initially androgen-dependent, become androgen-independent and refractory to hormone withdrawal therapy, presenting a significant problem in the treatment of the disease. It is not clear whether androgen-independent prostate tumours derive from pre-existing androgen-independent prostate cells or, more probably, from prostate cells that acquire the ability to proliferate in the absence of androgen. This ability is acquired by both AR-dependent and AR-independent mechanisms (Fig. 1).

Most androgen-independent prostate tumours express the AR (van der Kwast et al. 1991). It is activated in conditions of androgen deprivation by at least three mechanisms: overexpression as a result of gene amplification, cross-activation by agents other than androgens, and mutations that alter its specificity.

Gene amplification is probably the most common mutational mechanism for the AR. This event is very rare (0–1%) in untreated prostate tumours, but occurs in 22–30% of tumours that recur after endocrine treatment (Visakorpi et al. 1995, Koivisto et al. 1997, Bubendorf et al. 1999a).

Table 1 Genes involved in prostate cancer development.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Genes</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Overexpression inhibits metastasis occurrence</td>
<td>KAI1</td>
<td>Dong et al. 1995</td>
</tr>
<tr>
<td>Higher levels in androgen-independent than in androgen-dependent disease</td>
<td>bcl-2, p21, fibronectin, IGFBP-2, insulin receptor</td>
<td>McDonnell et al. 1992, Barettion et al. 1999, Stubbs et al. 1999, Bubendorf et al. 1999b</td>
</tr>
<tr>
<td>Lower levels in androgen-independent than in androgen-dependent disease</td>
<td>BTG-1, oncostatin-M</td>
<td>Chang et al. 1997, Bubendorf et al. 1999b</td>
</tr>
<tr>
<td>Gene amplification in advanced disease</td>
<td>androgen receptor, c-myc</td>
<td>Visakorpi et al. 1995, Bubendorf et al. 1999a</td>
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IGFBP, insulin-like growth factor binding protein.
failure of endocrine monotherapy in a prospective study of 92 patients (Palmberg et al. 2000), although this has not been confirmed by other studies. Tumours that developed androgen independence for reasons other than increased AR levels were less influenced by even lower androgen concentrations. Interestingly, one patient with high levels of AR gene amplification before hormonal therapy had a complete but temporary response to treatment, suggesting that this alteration is not always sufficient for androgen-independent behaviour (Edwards et al. 2001).

At least five non-androgen agents have been found to cross-activate the AR in the absence of androgen, by at least three distinct signalling pathways. Culig and co-workers (1994) showed that insulin-like growth factor (IGF)-I activates AR in the human prostate cancer cell line DU145, although this did not occur in LNCaP cells (Ueda et al. 2002). The difference may be attributable to variations between the signalling pathways in these cell lines. Forskolin (a plant lipid that stimulates cAMP) activates AR through the protein kinase A pathway.

**Figure 1** Molecular mechanisms involved in androgen independence. Evidence has been found for a number of possible mechanisms. AD, androgen-dependent; AI, androgen-independent; AR, androgen receptor; IGF-I, insulin-like growth factor I; IL-6, interleukin-6; TSGs, tumour suppressor genes.
Interleukin-6 (Ueda et al. 2002) and a related cytokine, oncostatin-M (Godoy-Tundidor et al. 2002) both activate AR in a ligand-independent manner, but through different pathways. Oncostatin-M may have an important role despite reduced levels of expression in androgen-independent cells in a xenograft model (Bubendorf et al. 1999b), as it also modulates the response of the AR to hydroxyflutamide, causing this anti-androgen to act as an agonist (Godoy-Tundidor et al. 2002). Such interactions may explain why, in certain patients, prostate-specific antigen (PSA) concentrations decrease after cessation of unsuccessful anti-androgen treatment. The transcription factor HER2/neu activates the AR independently of androgen (Craft et al. 1999, Yeh et al. 1999), and increases its response to low concentrations of androgen (Yeh et al. 1999). HER2/neu was overexpressed in androgen-independent derivatives of an androgen-dependent human prostate cancer xenograft studied by Craft and coworkers (1999). Such alterations in gene expression during endocrine treatment may allow the AR to be re-activated by concentrations of androgen that were previously too low.

Several groups have found evidence that the mitogen-activated protein kinase (MAPK) pathway is involved in cross-activation of the AR (Yeh et al. 1999, Ueda et al. 2002, Franco et al. 2003). Activation of the MAPK enzymes ERK1 and ERK2 was detected in 70% of prostate tumours with Gleason scores 8–10, and was highly significantly associated with increasing Gleason score (Gioeli et al. 1999). The MAPK pathway, among others, is activated by the Ras protein after its stimulation by a range of growth factors. The introduction of activated mutant forms of the H-Ras oncoprotein to androgen-dependent prostate cancer cells conferred hypersensitivity to low concentrations of androgen, androgen-independent growth in an animal model, and constitutive activity of MAPK enzymes (Bakin et al. 2003). Mutations that activate the ras family of oncoproteins frequently occur in some types of human cancer, but appear to be rare in prostate cancer (Carter et al. 1990, Gumerlock et al. 1991), indicating that other factors are responsible for the frequent MAPK enzyme activation in prostate tumours. However, a study of Japanese patients with prostate cancer found a greater frequency of ras mutations, suggesting that the Ras pathway may have a more significant role in this low-risk population than in Western countries (Anwar et al. 1992).

The clinical significance of AR mutations remains to be clarified. Estimates of the frequency of such mutations in prostate cancer vary, both before (Tilley et al. 1996, Marcelli et al. 2000) and after (Talpin et al. 1999, Wallen et al. 1999) hormonal treatment, ranging from 6% to 44%. This variation may partially be explained by methodological differences, but may also reflect genetic heterogeneity. One point mutation in the hormone-binding domain, found in the LNCaP human prostate cancer cell line, confers sensitivity to hormones other than androgens, including anti-androgens (Veldscholte et al. 1990). This mutation was reported to occur in five of 16 patients after combined androgen blockade with the anti-androgen, flutamide (Talpin et al. 1999), but in none of 36 hormone-refractory tumours tested by two other authors (Visakorpi et al. 1995, Koivisto et al. 1997).

Alternatively, in certain tumours the AR pathway may be bypassed altogether, by other mechanisms that regulate the balance of proliferation and apoptosis. Protein concentrations of the anti-apoptotic proto-oncogene, bcl-2, have been correlated with progression to androgen-independent status in human tumours, and bcl-2 mRNA was upregulated by testosterone withdrawal in rats (McDonell et al. 1992). Bcl-2 stable transfection enabled the androgen-dependent prostate cancer cell line, LNCaP, to survive androgen depletion in vitro and in vivo (Raffo et al. 1995) and protected the same cell line from apoptosis induced by melanoma differentiation associated gene-7 (mda-7)/IL-24 (Lebedeva et al. 2003). In contrast, overexpression of bcl-2 did not influence the viability of androgen-independent prostate cancer cells treated with mda-7/IL-24, which selectively induces apoptosis in a range of malignant cell types (Lebedeva et al. 2003).

c-myc mRNA levels were greater in prostate tumours than in benign prostate tissue (Fleming et al. 1986); furthermore, 11% of 62 metastatic prostate cancer specimens, but none of 223 primary tumours, showed c-myc gene amplification (Bubendorf et al. 1999a). However, another study of 130 untreated primary tumours detected c-myc gene amplification in 20% of cases, and found that it was significantly associated with higher Gleason score and risk of disease progression (Sato et al. 1999). The variation in reported frequency of c-myc gene amplification in primary tumours is possibly attributable to the selection of high-grade tumours in the last of these studies.

\( p21^{\text{WAF1/CIP1}} \), an effector of the p53 protein, negatively regulates the cell cycle, and its overexpression inhibits the proliferation of androgen-dependent and androgen-independent prostate cancer cells in vitro and tumorigenicity in vivo (Gotoh et al. 2003). However, it is expressed more frequently in androgen-independent than in androgen-dependent prostate cancer (Baretton et al. 1999, Fizazi et al. 2002). In a mouse model, androgen-independent prostate tumours lost \( p21^{\text{WAF1/CIP1}} \) expression after castration, but it was restored in androgen-independent tumours that subsequently relapsed (Fizazi et al. 2002). In addition, survival for patients with high \( p21^{\text{WAF1/CIP1}} \) levels was significantly shorter (Baretton et al. 1999). These
findings suggest that $p21^{WAF-1/CIP1}$ may exert a growth-stimulatory effect by a paracrine pathway (Fizazi et al. 2002) or that the G1/S cell cycle checkpoint is aberrantly regulated in some androgen-independent prostate tumours (Baretton et al. 1999).

$p53$ status may also influence responsiveness to androgens. In vitro experiments demonstrated that four $p53$ mutations common in prostate cancer each enabled the androgen-dependent prostate cancer cell line LNCaP to grow in an androgen-independent manner (Nesslinger et al. 2002).

Several studies have aimed to identify genes differentially expressed in androgen-dependent and androgen-independent prostate cancer. *Fibronectin*, a gene involved in cell adhesion, is expressed at higher levels in androgen-independent prostate cells (Stubbs et al. 1999). A novel putative cell cycle regulator and tumour suppressor, *BTG-1*, is expressed at reduced levels in an androgen-independent prostate cancer cell line, compared with its androgen-dependent parental cell line (Chang et al. 1997).

A cDNA microarray study on the androgen-dependent human prostate cancer xenograft, CWR22, and its androgen-independent derivatives showed that IGF binding protein (IGFBP)-2, *insulin receptor* and *IGF-II* were all overexpressed in androgen-independent conditions (Bubendorf et al. 1999b). IGFBP-2 overexpression was validated by a tissue microarray method (Bubendorf et al. 1999b). It is worth noting, as mentioned previously, that IGF-I can activate the AR in the absence of androgen (Culig et al. 1994). In addition, prostate cancer risk has been correlated to IGF-I concentrations in plasma (Chan et al. 1998), and IGFBP-3 concentrations are lower in malignant than in normal prostate (Dhanasekaran et al. 2001). The proto-oncogene *hK2* and hepsin have been identified by microarray experiments and are discussed later.

**Microarray studies of prostate cancer gene expression**

Microarray technology (Fig. 2) has provided a powerful tool for large-scale studies of changes in gene expression. This technique involves the spotting of up to several thousand specific cDNAs or oligonucleotides on a single slide or chip in a known arrangement, and hybridising the slide to the samples. There are a number of approaches to microarray analysis. In one approach, the samples are prepared by extracting mRNA from the cells or tissues of interest (such as prostate cancer and normal prostate), labelling them with different fluorescent dyes, and pooling them. After unbound sample has been removed, fluorescence at the two relevant wavelengths is measured at each spot on the slide. Analysis of the resulting data reveals the levels of expression of the genes in the tissues tested. Appropriate and representative sample selection is of crucial importance, and differences in gene expression revealed by microarrays must be validated by other techniques for measuring mRNA and protein concentrations. cDNA or oligonucleotide arrays can be complemented by tissue microarrays, in which large numbers of tissue sections are arrayed on a single slide and challenged with an antibody of interest (Dhanasekaran et al. 2001).

As with other methods, in microarray studies also, genes involved in cell proliferation are clearly important (Stamay et al. 2001, LaTulippe et al. 2002). c-myc mRNA levels were greater in prostate tumours than in normal prostate tissue (Dhanasekaran et al. 2001), as found previously (Fleming et al. 1986). The proto-oncogene *PIM1* was upregulated in prostate cancer compared with normal prostate (Dhanasekaran et al. 2001). However,
Microarray production
Several thousand cDNA or oligonucleotide sequences spotted on slide

Sample preparation
mRNA extracted from tissues or cells of interest and labelled with fluorescent dyes

Hybridisation and washing
Fluorescence measurement

Microarray results
Expression profile of selected genes in tissues of interest

Validation
of microarray results by further analysis of relevant tissues

Northern blotting
Reverse-transcriptase PCR
Tissue microarray (TMA)

New diagnostic and therapeutic targets

Figure 2 From microarrays to new diagnostic and therapeutic targets. Microarrays allow the analysis of patterns of expression of large numbers of genes in tissues of interest, such as advanced prostate cancer and localised prostate cancer. Visual representations of data show spots of different colours and intensities for genes expressed in either or both tissues at differing levels.
among patients, lower PIM1 levels were strongly associated with an increased risk of relapse after radical prostatectomy (Dhanasekaran et al. 2001). The three genes MYBL2, cyclin E and CDC2, which are involved in stimulating mitosis through the same pathway, are all upregulated in metastatic prostate cancer compared with non-recurrent primary tumours (LaTulippe et al. 2002). Dhanasekaran and coworkers (2001) identified a number of genes, including metastasis-associated-1 (MTA-1), that showed changes in expression profile in metastatic prostate cancer when compared with localised cancer. A cDNA microarray study showed that IGFBP-2 was upregulated in metastatic prostate cancer compared with androgen-dependent disease (Bubendorf et al. 1999b). The role of IGF-I in androgen independence is discussed above.

Varambally et al. (2002) used microarrays to characterise benign prostate, clinically localised prostate cancer and metastatic prostate cancer and, of 55 genes upregulated in metastatic disease compared with localised disease, EZH2 (enhancer of zeste homologue 2) showed the greatest magnitude of overexpression. This gene encodes a homologue of a Drosophila transcriptional repressor gene. Its overexpression in the prostate cell line RWPE resulted in repression of a wide range of genes, at least some of which appear to function in prostate cancer proliferation, as small interfering RNA (siRNA) down-regulation of EZH2 in vitro resulted in growth inhibition of prostate cancer cells (Varambally et al. 2002). Subsequently, prostate tumours that were both EZH2-positive and E-cadherin-negative were shown to be significantly more likely to recur (Rhodes et al. 2003).

Fatty acid synthase (Dhanasekaran et al. 2001) and another fatty acid metabolism enzyme, human z-methylacyl-CoA racemase (AMACR) (Xu et al. 2000b), were also shown to be overexpressed in prostate tumours, and were significantly overexpressed in a meta-analysis of four studies (Rhodes et al. 2002). Tissue microarrays showed that, as was the case with hepsin, overexpression of AMACR was greater in localised tumours than in metastases, possibly as a result of dedifferentiation (Kuefer et al. 2002).

Microarray experiments have revealed some trends that would be difficult to identify by other techniques. A meta-analysis by Rhodes and coworkers (2002) of four microarray studies of prostate cancer showed that biosynthesis of polyamines and of purines was consistently dysregulated in prostate cancer. Almost 25% of the genes found by LaTulippe and coworkers (2002) to be most overexpressed or underexpressed during the progression of prostate cancer were associated with regulation of gene expression.

Microarray experiments have also identified potential new biochemical markers for prostate cancer. Hepsin, a transmembrane serine protease, was expressed more highly in tumour than in normal prostate in all four studies in this meta-analysis (Rhodes et al. 2002). Its overexpression was validated by RT-PCR (Welsh et al. 2001) and tissue microarray (Dhanasekaran et al. 2001). Stamey and coworkers (2001) investigated differences between benign prostatic hyperplasia and Gleason grade 4/5 prostate cancer using cDNA arrays and found that, of almost 7000 genes tested, hepsin showed most upregulation in cancer. The greatest levels of hepsin were found in high-grade prostatic intraepithelial neoplasia (PIN), and levels were reduced in metastatic samples compared with localised disease samples (Dhanasekaran et al. 2001). Tumours in which hepsin was absent or present in low levels were more likely to relapse after radical prostatectomy, although the association of reduced PIM1 with relapse was still stronger (Dhanasekaran et al. 2001).

Conclusion
This review represents a summary of the rapidly increasing knowledge of the molecular pathology of prostate cancer. Alterations in expression or function of numerous genes involved in key aspects of carcinogenesis, including cell cycle regulation, steroid hormone metabolism and regulation of gene expression, are associated with disease progression. Metastatic (Dhanasekaran et al. 2001, LaTulippe et al. 2002) and androgen-independent (Bubendorf et al. 1999b) prostate cancer have been specifically examined. Androgen independence can be acquired by a number of molecular mechanisms, including cross-activation by agents such as IGF-I (Culig et al. 1994). Potential new molecular markers such as hepsin (Rhodes et al. 2002) have been identified. These and other identified and novel genes implicated in the disease merit more investigation of their clinical relevance. The emergence of microarray technology allowing simultaneous analysis of the expression of thousands of genes, and hundreds of tissue specimens, should facilitate rapid progress in identifying key molecular players in the development of prostate cancer.

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