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Molecular markers of prostate cancer outcome

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Abstract

Molecular markers have the potential to serve not only as prognostic factors but may be targets for new therapeutic strategies and predictors of response in a range of cancers. Prostate cancer development and progression is predicated on a series of genetic and epigenetic events within the prostate cell and its milieu. Within this review, we identify candidate molecules involved in diverse processes such as cell proliferation, death and apoptosis, signal transduction, androgen receptor (AR) signalling, cellular adhesion and angiogenesis that are linked to outcome in prostate cancer. Current markers with potential prognostic value include p53, Bcl-2, $p16^{INK4A}$, $p27^{Kip1}$, c-Myc, AR, E-cadherin and vascular endothelial growth factor. Evolving technology permits the identification of an increasing number of molecular markers with prognosis and predictive potential. We also review the use of gene microarray analysis in gene discovery as a means of identifying and cosegregating novel markers of prostate cancer outcome. By integrating selected markers into prospective clinical trials, there is potential for us to provide specific targeted therapy tailored for an increasing number of patients.

Keywords: Prostate cancer; Prognosis; Molecular markers; Oncogenes; Tumor suppressor genes

1. Introduction

The molecular biology of prostate cancer and its progression is characterised by aberrant activity of several regulatory pathways, both within the prostate cells and in the surrounding tissue. These pathways can be grouped broadly into apoptosis, androgen receptor (AR) signalling, signal transduction, cell cycle regulation, cell adhesion and cohesion, and angiogenesis (Table 1). Variations at the DNA, RNA and/or protein levels of molecules involved in these pathways are all potential candidate markers of prognosis and therapeutic response. Detailed cohort studies have delineated the clinical and pathological factors that predict outcome for men diagnosed with prostate cancer on biopsy and after a variety of treatments for clinically localised disease. On this basis, any new prognostic marker must be measured in the context of accepted predictors of prostate cancer recurrence and death. These are: clinical or pathological disease stage; surgical margin involvement; Gleason score or grade; and serum prostate-specific antigen (PSA) concentration at diagnosis [1–8]. For a prognostic marker to be of use, it must provide value additional to, and possibly independent of, that are provided by these factors. However, molecular markers are not only important because of potential relationships with outcome, they also provide putative targets for molecular-based intervention for

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Table 1
Summary of molecular aberration in prostate cancer

Process	Key molecules/markers	Selected references
Apoptosis	p53, Bcl-2	[128–133,174]
Androgen receptor signalling	AR, possible alternate signal transduction pathways	[194,199,205,217,231,232,238,369]
Signal transduction	Epidermal growth factor receptor family	[240,241]
Cell cycle regulation	c-Myc, $p16^{INK4A}$, $p27^{KIP1}$, pRb, apoptotic index, Ki67	[42,47,48,54,119,122]
Cell adhesion and cohesion	E-cadherin, α-catenin, metalloproteinases, chondroitin sulphate	[285–287,289,317,318,331,370–374]
Angiogenesis	VEGF, VEGF receptors, nitric oxide	[139,141,322,323,326]

AR, androgen receptor; VEGF, vascular endothelial growth factor.

the cancer type concerned. From this perspective, while an association with adverse outcome might suggest a key role for a given molecule in the disease state, it does not mean that markers that are not prognostic are of no use. For example, a marker that is present in a large number of prostate cancers and might be targeted therapeutically is likely to be of considerable interest and utility. This review will largely focus on molecular markers of outcome clinically localised prostate cancer and will present data predominantly from series of patients treated with radical prostatectomy (RP), with supplemental data from series involving patients treated with other modalities and with advanced disease where relevant.

In taking a translational research approach to study cancer outcome, one can take a candidate gene approach, in which known genes of putative importance in a particular cancer are assessed in a series of tumour samples and compared with clinico-pathological factors including outcome. Alternatively, one can use a variety of techniques in an attempt to discover new genes that may be important in the cancer concerned. Techniques designed to detect chromosomal abnormalities in prostate cancer have identified a number of potential candidate molecules for evaluation in prostate cancer (Table 2). More recently, the ability to assay tumour tissue using cDNA and oligonucleotide arrays with identified sequences for many thousands of molecules has expanded the scope and number of such markers inestimably [9,10]. Candidate molecules identified in these ways can then be evaluated in tumour samples. The construction of tumour tissue microarrays, in which cores of multiple different cancers are assembled in one paraffin block and can be stained for protein expression on a single slide allows rapid assessment and validation of these markers [11]. In addition, selection of overexpressed molecules by cellular localisation and function can lead to the development of new markers for cancer in blood and other body fluids [12]. The search for prognostic tissue markers is not an end in itself because, apart from providing information on outcome, it may also lead to advances in diagnostic methods, delineate therapeutic targets and identify other related molecules important in cancer development and progression.

2. Cell proliferation and death in prostate cancer

The essential elements in the progression of any hormone-dependent cancer are deregulated cell proliferation, avoidance of apoptosis, resistance to hormonal control and metastasis. Each of these important biological events has important clinical correlates (Table 3).

Increased proliferation index, whether measured by Ki67, proliferating cell nuclear antigen (PCNA) or bromo-deoxyuridine incorporation, correlates with the presence of advanced stage disease [13,14] or increased tumour grade [14–16]. Ki67 index is independently predictive of outcome in patients with clinically localised disease treated with RP [14,15,17–19] with radiotherapy [20] and patients being observed [21]. Recurrent tumours have Ki67 indices approximately double that of the primary tumour [22,23].

Several studies [24–26] have identified increased apoptotic index (ApI), a measure of the number of apoptosing cells within a prostate cancer, as adversely prognostic. One study found increased ApI was independently predictive of outcome following RP, whereas in the same set, p53 and bcl-2 were not [26]. Unfortunately, these studies have been undertaken in relatively small groups, making the wider application of reported results problematic, but nonetheless pointing to the potential importance of apoptosis in prostate cancer.

3. Cell cycle regulation

Genetic aberrations in the control of G_1- to S-phase progression in the cell cycle are present in virtually all human cancers. Progression through the G_1/S-phase checkpoint is controlled by the sequential transcriptional activation of cyclin genes, and the consequent transient accumulation and activation of a sequence of cyclin/cyclin-dependent kinases (CDK) complexes, resulting in hyperphosphorylation of the retinoblastoma gene product pRb [27] (Fig. 1). There have been significant recent advances in knowledge about the molecular basis of cell cycle control due to the discovery and functional analysis of the cell cycle regulatory cyclins, CDKs and CDK inhibitors [28,29]. A number of endogenous inhibitors of CDK catalytic activity (CDIs) exist with

Table 2
Summary of chromosomal abnormalities described in prostate cancer selected for prevalence and prognostic potential

Chromosomal locus	Possible product	Percentage	Disease stage	Therapeutic status: hormonal therapy	References
1q gain		52	Metastases	Androgen-independent	[117]
2q14 gain	Bin 1	40	Metastases	Naïve	[375]
5q loss		39	Metastases	Androgen-independent	[117]
6q14-21 loss		25	Localised	Untreated	[376]
6q loss		39	Metastases	Androgen-independent	[117]
7p gain	EGF receptor	8	Localised	Untreated	[377]
8p loss	Undefined tumour suppressor genes	80	Metastases	Androgen-independent	[117]
8p loss		33	Localised	Untreated	[378]
		65	Locally recurrent	Untreated	
		83	LN metastases	Untreated	
8p12-12 LOH		63	PIN		[379,380]
		90.6	Localised	Untreated	
8p22 loss		62	Localised T3N0M0	Untreated	[119]
8p22 loss		63	Metastases	Untreated and androgen-independent	[381–384]
8q gain	c-Myc	33–54	Localised T3N0M0	Untreated	[119,385,386]
8q gain		57–85	Metastases	Untreated and androgen-independent	[116,117,386]
9q21 point mutations	$p16^{INK4A}$ $p15^{INK4B}$ $p19^{ARF}$	0	Localised	Untreated	[65]
9q21 LOH			Localised		[387]
		15	T2N0M0	Untreated	
		46	T3N0M0	Untreated	
9q21 LOH		20	Localised	Untreated	[65]
		46	Metastases	Untreated	
9q21 methylation		13	Localised	Untreated	[65]
		8	Metastases	Untreated	
10p15 loss	Kruppel-like factor 6 (KLF6 or Zf9)	15–77	Localised	Untreated	[388,389]
10q loss		30	Localised	Untreated	[390,391]
10q21 LOH	ANX7	35	Localised	Untreated	[392]
10q23 loss	PTEN	50	Metastases	Untreated and androgen-independent	[117,393]
10q23.3 LOH	PTEN	14	Localised	Untreated	[117,394]
		43	T2-3,N+	Untreated	
		63	Metastases	Androgen-independent	
10q24 LOH	LAPSER1	55	Localised	Untreated	[383,391]
11p gain	H-ras, Kai-1	52	Metastases	Androgen-independent	[117]
13q14.2 loss	pRb or chromosome condensation 1-like (CHC1-L)	33	Localised	Untreated	[50,395]
13q loss		75	Metastases	Untreated and androgen-independent	[51,117]
16q loss	E-cadherin	30	Localised	Untreated	[390]
16q23.1-24 loss	E-cadherin	55	Metastases	Untreated and androgen-independent	[117,383,396]
17q11-12 gain	HER-2/neu, erbB2	30	Metastases	Untreated and androgen-independent	[117,386]
17p loss	p53	18	Localised	Untreated	[156]
17p13.1 loss	p53	50	Metastases	Untreated and androgen-independent	[117]
Xq11-13	Androgen receptor	22–36	Metastases	Androgen-independent	[118,123,202,386]

LN, lymph node; PIN; prostatic intraepithelial neoplasia; LOH, loss of heterozygosity; EGF, epidermal growth factor.

varying but overlapping specificities [29–31]. $p27^{Kip1}$ inhibits cyclin E/Cdk-2 activity but also binds to D-type cyclins. $p21^{WAF1/CIP1}$ is, in part, p53-regulated, and binds to a range of cyclin/CDK complexes, including those with cyclins D1 and E, and acts as an inhibitor in some settings but also act as an adaptor protein for cyclin/CDK assembly in others [32–34]. $p16^{INK4A}$ inhibits Cdk-4 and Cdk-6 catalytic activity [35,36]. c-Myc has stimulatory effects on cell cycle progression at least in part through interaction with components of the $p27^{Kip1}$/cyclin E/Cdk2 complex [37]. A series of molecules regulate the physiological effect of c-Myc: Mad, Max and Mxi1, which through heterodimer interaction regulate the transcription and cell cycle regulatory activity of c-Myc [38]. Interestingly, c-Myc and Mad interact to regulate AR-mediated transcription [39,40].

Table 3
Summary of sequential proliferative and apoptotic changes in prostate carcinogenesis and prostate cancer progression [108,397–399]

Phase/stage of prostate cancer	Doubling time Mean (d)	Proliferative rate relative to preceding phase or stage	Apoptotic rate relative to preceding phase or stage
Normal prostate tissue	500	NA	NA
Low-grade prostatic intraepithelial neoplasia	150	Increased	Unchanged
High-grade prostatic intraepithelial neoplasia	56	Increased	Increased
Localised prostate cancer	50	No changed	Decreased
Metastatic prostate cancer: lymph nodes	33	Increased	Increased
Metastatic prostate cancer: bone	54	Increased	Increased
Hormone-responsive prostate cancer	NA	Decreased	Increased
Prostate cancer – at onset of hormone-resistance	NA	No change compared with untreated, but increased compared with hormone responsive	Increased compared with untreated, but decreased[a] compared with hormone responsive
Prostate cancer – hormone-resistant – agonal	NA	No change	Decreased[a]

NA, not applicable.
[a] Decrease may relate to local or general nutrient delivery.

These CDIs and the functionally associated *pRb* genes are all tumour suppressor genes of potential significance in prostate cancer. Each of the cyclins as well as c-myc are potential oncogenes in prostate cancer.

3.1. Retinoblastoma protein

When retinoblastoma protein (pRb), is present in a hypophosphorylated form during G_0 and G_1 phases it serves to inhibit cell cycle progression into S-phase [27]. Phosphorylation of pRB by cyclin D/CDK complexes in G_1-phase inactivates pRb and is essential for progression through the cell cycle. Hence, pRb has a central role in cell cycle regulation.

Retinoblastoma gene mutations correlate closely with loss of retinoblastoma protein expression, as measured by immunohistochemistry (IHC) [41–43]. Loss of pRb expression, as measured by IHC, is prognostic in endometrial, non-small cell lung and bladder cancer [44–46]. Despite this, there are few sizeable series of prostate cancer in which role of pRb expression as a prognostic indicator has been examined. Loss of pRb expression has

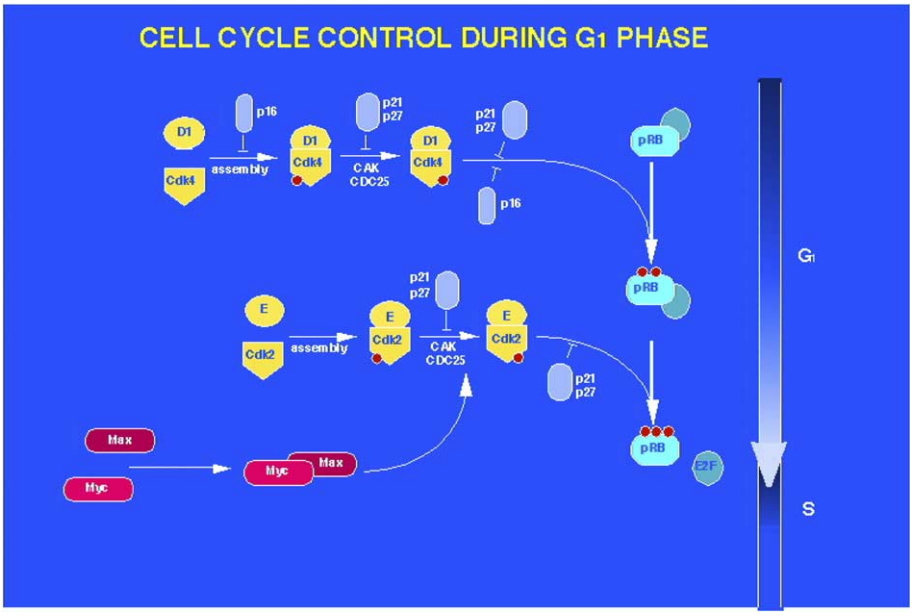

Fig. 1. Cell cycle progression during G_1-phase. A simplified model of interactions between some of the molecules involved in G_1-phase of the cell cycle. Progression through G_1-phase of the cell cycle requires the activity of cyclin D1 and cyclin E and their catalytic partners cyclin-dependent kinase subunits, Cdk4/6 and Cdk2, respectively. The active cyclin-Cdk complexes bind to and phosphorylate pRb. In its unphosphorylated form, pRb sequesters transcription factors of the E2F family and pRb phosphorylation releases E2F. This allows transcription of essential E2F responsive genes and cell cycle progression from G_1- to S-phase. The cell cycle is further regulated by two families of cyclin-dependent kinase inhibitors, the INK4 family (such as $p16^{INK4A}$) and the Cip/Kip family ($p21^{WAF1/CIP1}$, $p27^{Kip1}$). C-myc exerts a positive effect on cell cycle progression through cyclin E and p27.

Table 4
Cell cycle markers and prostate cancer outcome

Molecule	Treatment	Cohort size	Stage	Dichotomising level for marker expression	Effect on prostate cancer outcome	Special notes	References
Retinoblastoma protein					Summary: inconclusive		
Decrease or loss	RP	71	T1-2,N0	NA	Poorer survival		[400]
	RP	118	T1-2,N0	NA	Nil		[52]
Cyclins					Summary: inconclusive		
Increased cyclin D1	RP	140	T1-3,N0-1	>5% nuclear accumulation	Nil		[58]
Increased cyclin D	RP	213	T1-3,N0-1		Prognostic but loses significance with other factors	Antibody cocktail for all cyclin Ds	[57]
Increased cyclin A	RP	213	T1-3,N0-1		Independently prognostic		[57]
Increased cyclin A	RP	28	T1-2,N0	Index with Ki67	Nil		[61]
Increased cyclin B	RP	28	T1-2,N0	Index with Ki67	Nil		[61]
Increased cyclin E	RP	28	T1-2,N0	Index with Ki67	Nil		[61]
$p16^{INK4A}$					Summary: Prognostic		
Increased $p16^{INK4A}$	RP	88	T1-3,N0-1	>5% nuclear accumulation	Prognostic	Immunohistochemistry confirmed by in situ hybridisation	[71]
	RP	104	T1-3,N0-1	NA	Independently prognostic	CDK4 not elevated	[72]
	RP	206	T1-3,N0-1	>1% nuclear accumulation	Independently prognostic	Elevation in HGPIN also prognostic	[54]
Increased $p21^{WAF1/CIP1}$					Summary: probably prognostic		
	RP	213	T1-3,N0-1	>5% nuclear accumulation	Independently prognostic	Independent of p53	[401]
	RP	88	T1-3,N0-1	>5% nuclear accumulation	Prognostic	Independent of p53	[79]
	RP	60		>5% nuclear accumulation	Independently prognostic	Independent of p53	[402]
	RP	86	T1-4,N0-1	>10% nuclear accumulation	Nil		[115]
Decreased $p27^{Kip1}$					Summary: probably prognostic		
	RP	113	T1-3,N0-1	<25% nuclear accumulation	Independently prognostic	Effect greatest in patients treated with neoadjuvant hormonal therapy	[110]
	RP	96	T3, N0-1	10, 50%	Prognostic		[111]
	RP	86	T1-3,N0-1		Independently prognostic		[113]
	RP	95	T1-4,N0-1	<10% nuclear accumulation	Independently prognostic		[114]
	RP	138	T1-3,N0-1	<50% nuclear accumulation	Nil		[403]
	RP	73	T1-3,N0-1	Variety	Nil		[404]
C-MYC amplification					Summary: probably prognostic		
	RP	114	T3	*C-MYC* copy number by FISH	Prognostic	Limited by technical requirements for analysis using FISH	[119]
	RP	52	T2-3,N1-3	*C-MYC* copy number by FISH	Prognostic		[172]

HGPIN, high-grade prostatic intraepithelial neoplasia; NA, not applicable; RP, radical prostatectomy; FISH, fluorescence in situ hybridisation.

been reported in as many as 100% and as few as 1% of prostate cancers, while loss of at least one *Rb* allele is reported in 20–60% of tumours [41,47–51]. Generally, there is progressive loss of pRb expression with increasing prostate cancer grade and stage. Localised prostate cancers show loss of pRb expression in 1–45% of cases [47,49,52], while the number rises to 20–60% in advanced prostate cancer [49,53]. Whether pRb expression portents relapse and predicts survival is a matter of debate (Table 4). Data from the St. Vincent's Campus Prostate Cancer Group suggests pRb expression has limited prognostic value but may warrant further study [54].

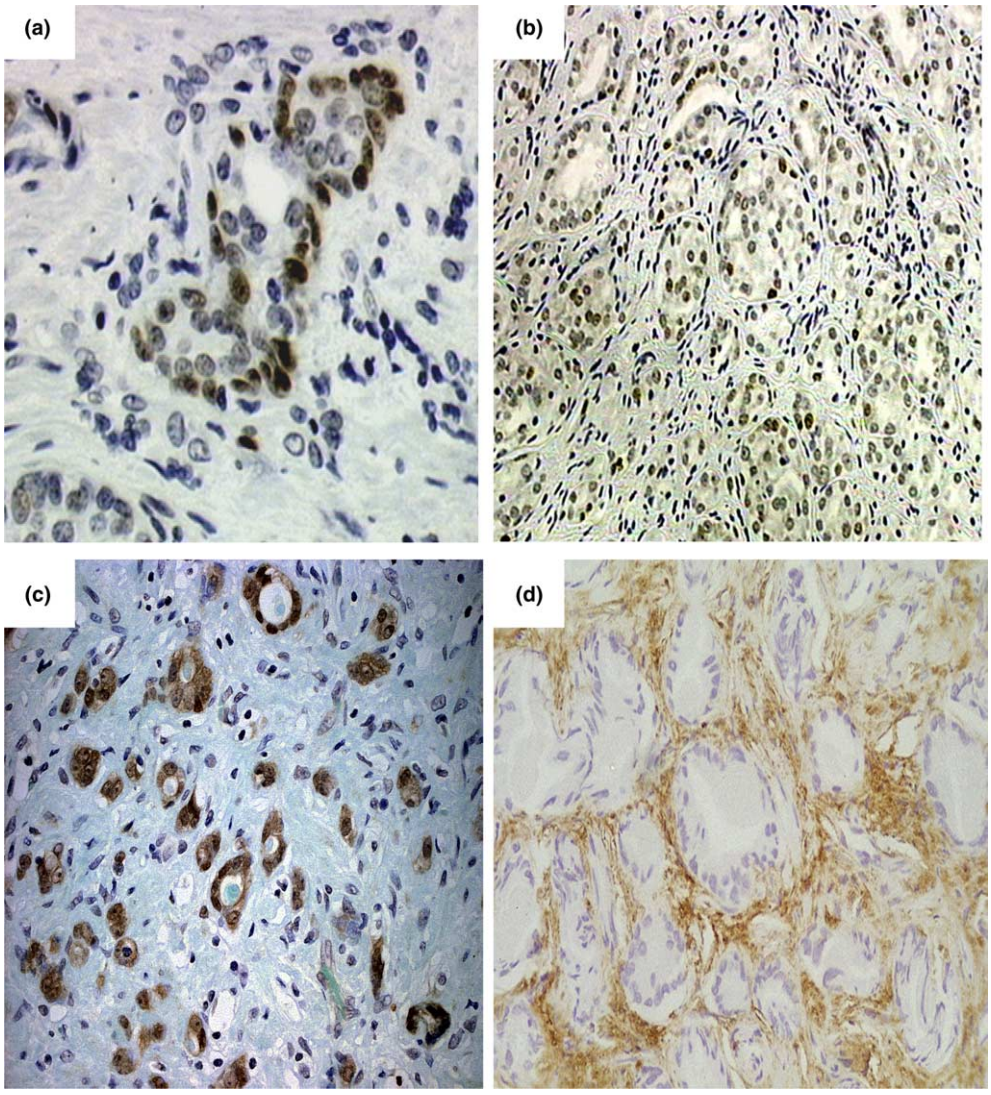

Fig. 2. Representative photomicrographs of prostate cancer stained immunohistochemically for: (a) p53, showing a cluster of cells with nuclear accumulation (400×); (b) p53, showing more diffuse nuclear staining for p53 (200×); (c) p16INK4a overexpression in the nuclei and cytoplasm (400×); and (d) chondroitin sulphate expression in the stroma adjacent to prostate cancer forming circumscribed glands (400×). From Quinn and colleagues [133], Henshall and colleagues [54] and Ricciardelli and colleagues [317] with permission from the American Association for Cancer Research.

3.2. Cyclins

The D-type cyclins include three known variants (D1, D2 and D3) with 57% total homology and 78% homology in the functional region known as the 'cyclin box' [55]. Despite this close homology, each is encoded by distinct genes: *CCND1*: 11q13, *CCND2*: 12p13 and *CCND3*: 6q21 [55]. Functionally, the three subtypes of D cyclin behave similarly. Cyclin D1 overexpression is a common molecular aberration in many human cancers. However, in localised prostate cancer the presence of CCND1 amplification and cyclin D1 overexpression is reported as being rare and probably occurs in less than 5% of cases [56; Henshall and colleagues, data not shown]. However, several authors have reported a higher rate of cyclin D1 or overall cyclin D expression in localised prostate cancer with apparent prognostic effect [57] (Table 4). There are currently no published studies of cyclin D2 or D3 expression and outcome in prostate cancer. Two studies have addressed cyclin A overexpression, with one finding it to have independent prognostic effect [57,58] (Table 4). Cyclin E overexpression is prognostic in a number of tumours, either in concert with decreased p27 expression, or alone [34,59,60]. Work on cyclin E expression in prostate cancer is very limited, with a single published study [61] (Table 4). Further study of cyclin expression as a prognostic parameter in prostate cancer is warranted.

3.3. $p16^{INK4A}$

The tumour suppressor gene *INK4A* located at 9q21 is frequently inactivated in human cancers, including melanoma, pancreatic cancer and squamous cell carci-

noma of the head and neck [36]. The product of *INK4A* was first identified as a 16-kDa molecule that inhibited the effect of CD*K4*, hence the designation $p16^{INK4A}$ [62]. The two most common mechanisms for loss of $p16^{INK4A}$ function are homozygous deletion and loss of transcription due to hypermethylation of the $p16^{INK4A}$ promoter [63]. Hypermethylation of $p16^{INK4A}$ was detected in the androgen-independent prostate cancer cell line PC-3 [63] and bi-allelic inactivation of $p16^{INK4A}$ by a combination of gene deletion and methylation have been reported in a small subset of tumours [64–67]. Other studies have failed to detect $p16^{INK4A}$ gene mutations in small series of primary prostate cancers [68–70].

The available data on *INK4A* gene status and/or $p16^{INK4A}$ expression in relation to prostate cancer outcome are limited to three studies, which despite some methodological differences deliver the same conclusion: $p16^{INK4A}$ *overexpression* is adversely prognostic. These case series are summarised in Table 4 [54, 72:1408]. The studies to find $p16^{INK4A}$ overexpression adversely prognostic in prostate cancer have done so in the presence of elevated INK4A exon 1α transcripts [71], in the absence of a concurrent elevation of CDK4 expression in transition from benign to malignant prostate tissue [72] and without correlation with loss in retinoblastoma protein expression [54] (Figs. 2(c) and 4). Overexpression of $p16^{INK4A}$ in these tumours may indicate the presence of an inactive pRB as in several other human cancers [35] and elevated *INK4A* exon 1α transcript expression is consistent with a downstream feedback effect on $p16^{INK4A}$ gene transcription [73]. The question as to how prostate cancer cells escape the effects of increased $p16^{INK4A}$ expression in braking cell cycle progression is important. *In vitro* studies suggest increased levels of $p16^{INK4A}$ are important in inducing prostate epithelial cell senescence and that abrogation of the $p16^{INK4A}$/Rb pathway is required for these cells to bypass senescence and undergo immortalisation as part of tumourigenesis [74]. Hence, elevation of $p16^{INK4A}$

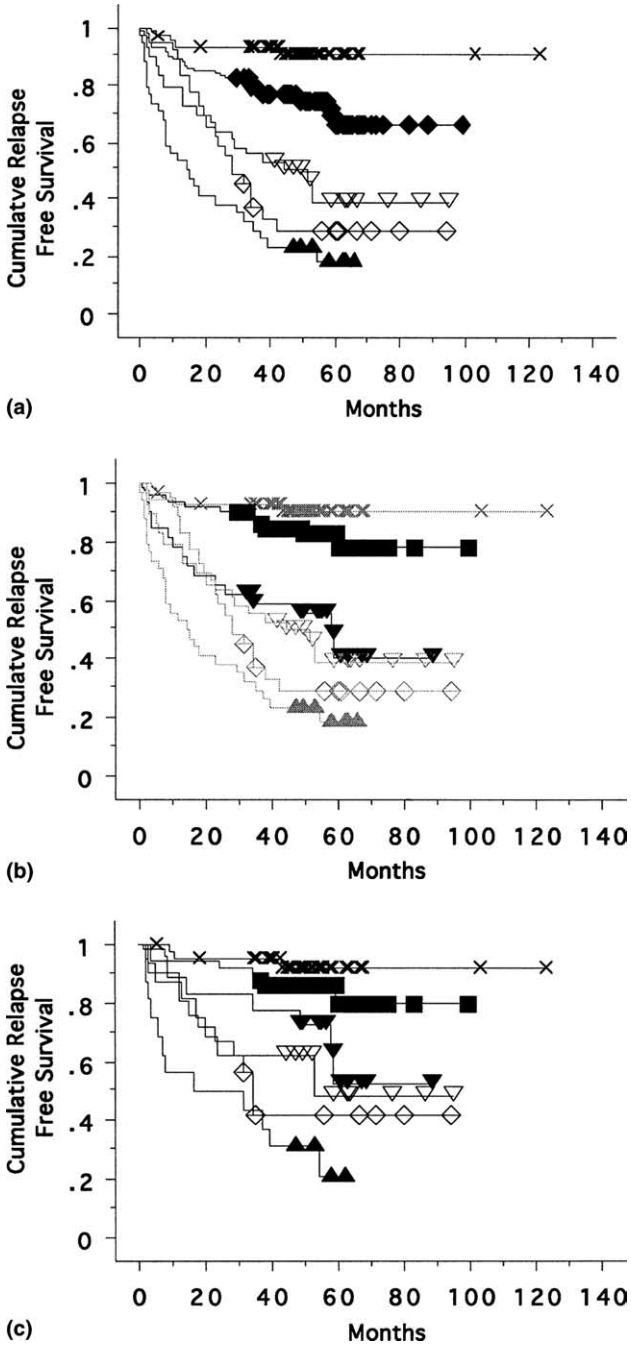

Fig. 3. p53 nuclear accumulation and relapse-free survival. (a) Relapse-free survival for patients with clinical localised prostate cancer treated with radical prostatectomy (RP) with or without neoadjuvant or adjuvant therapy categorised by p53 immunohistochemistry (IHC) score into strata: 0 (×); >0 to <2% (◆); 2 to <5% (▼); 5 to < 20% (◇); 20% (▲) of immunoreactive nuclei. Survival curves were generated according to the Kaplan–Meier method, and statistical comparisons were made by use of the log-rank method ($n = 263$; overall log-rank, $P < 0.0001$; intergroup log-rank: 0 *versus* >0 to <2%, $P = 0.01$; >0 to <2% *versus* 2 to <5%, $P = 0.002$; 2 to <5% *versus* 5% to <20%, $P = 0.24$; and 5% to <20% *versus* >20%, $P = 0.19$). (b) Relapse-free survival for patients with clinically localised prostate cancer treated with RP with or without neoadjuvant or adjuvant therapy categorised by p53 IHC strata incorporating p53 cluster status: 0 (×); >0 to <2% and cluster negative (■); >0 to <2% and cluster positive (▼); 2 to <5% (▽), 5 to <20% (◇); 20% (▲) of immunoreactive nuclei ($n = 263$, overall log-rank, $P < 0.0001$; intergroup log-rank: 0 *versus* >0 to <2%, cluster negative, $P = 0.19$; >0 to <2%, cluster negative *versus* >0 to <2%, cluster positive, $P = 0.0004$; >0 to <2%, cluster positive *versus* 2 to <5%, $P = 0.73$; 2 to <5% *versus* 5% to <20%, $P = 0.24$; 5% to <20% *versus* 20%, $P = 0.19$; and 2 to <5% *versus* 20%, $P = 0.009$). (c) Relapse-free survival for patients with localised prostate cancer treated with RP alone by p53 IHC strata as for (b), demonstrating a similar relationship between p53 score and relapse in this subset of patients ($n = 164$; overall log-rank, $P < 0.0001$; all intergroup log-rank P values were not significant except that for >0 to <2%, cluster negative *versus* >0 to <2%, cluster positive: $P = 0.048$). From Quinn and colleagues [133] with permission from the American Association for Cancer Research.

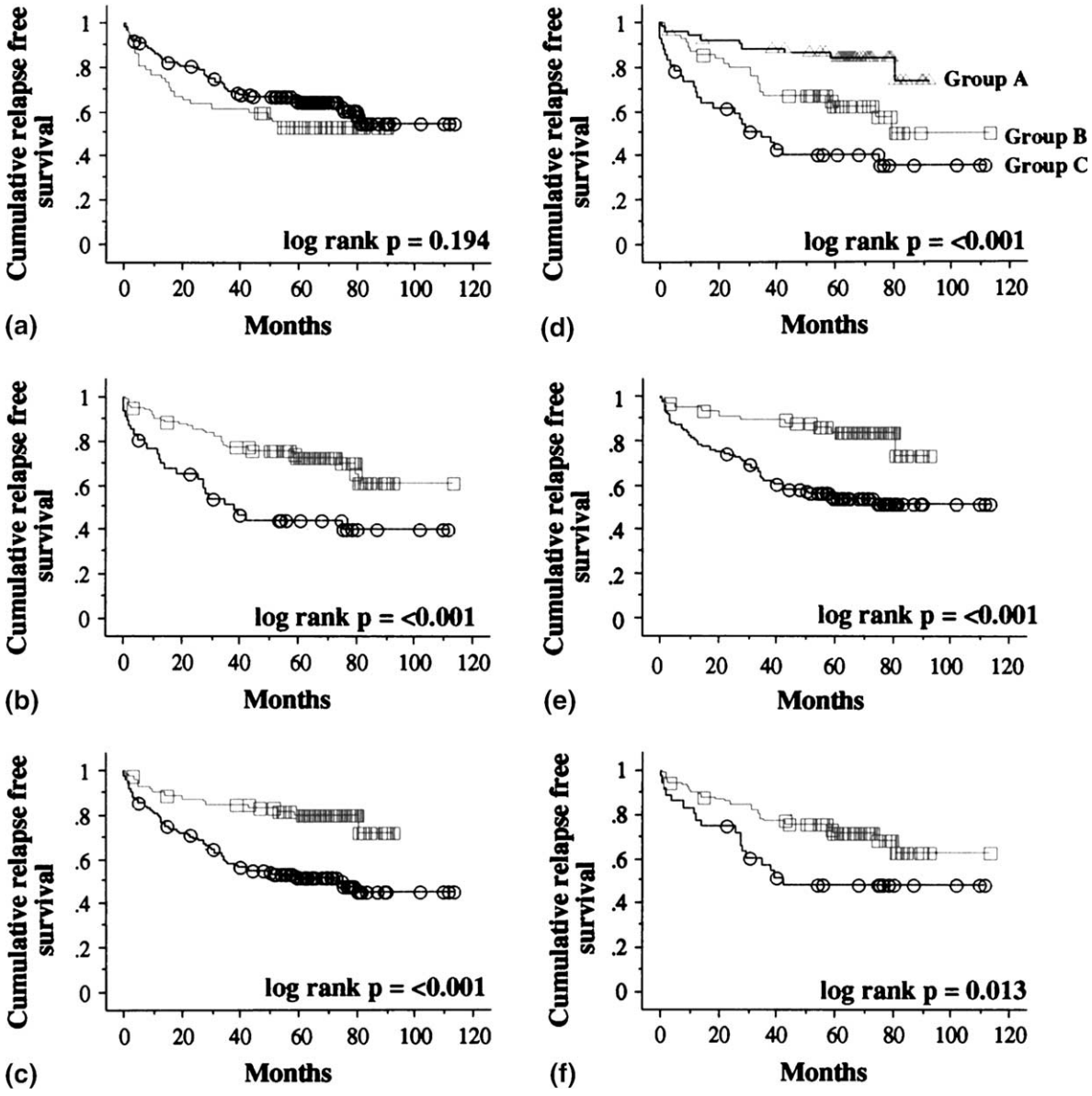

Fig. 4. $p16^{INK4A}$ expression and relapse-free survival. Relapse-free survival for patients with clinically localised prostate cancer treated with radical prostatectomy (RP) categorised as follows: (a) Histological diagnosis of high-grade prostatic intraepithelial neoplasia (HGPIN) (log rank, $P = 0.194$) ○, HGPIN present in RP;□, HGPIN absent in RP. (b) $p16^{INK4A}$ strata in areas of HGPIN (log rank, $P < 0.001$). □, 1% $p16^{INK4A}$ nuclear immunoreactivity; >1% $p16^{INK4A}$ nuclear immunoreactivity. (c) $p16^{INK4A}$ strata in areas of cancer (log rank, $P < 0.001$). □, ⩽ 1% $p16^{INK4A}$ nuclear immunoreactivity;○, >1% $p16^{INK4A}$ nuclear immunoreactivity. (d) Combined $p16^{INK4A}$ strata in areas of HGPIN and cancer in the same specimen [*Group A*, low $p16^{INK4A}$ expression (1%) in HGPIN and cancer; *Group B*, high $p16^{INK4A}$ expression (>1%) in cancer and low in HGPIN; and *Group C*, high $p16^{INK4A}$ expression (>1%) in HGPIN and cancer (overall log rank, $P < 0.001$). Differences between the three groups were significant by log-rank calculation between *Group A* and *B*, $P = 0.032$; between *Group B* and *C*, $P = 0.018$. (e) After exclusion of the patients treated with neoadjuvant hormonal therapy (NHT), Kaplan–Meier analysis of $p16^{INK4A}$ expression in 168 patients categorised by $p16^{INK4A}$ strata in areas of cancer (log rank, $P < 0.001$). □, ⩽ 1% $p16^{INK4A}$ nuclear immunoreactivity;○, >1% $p16^{INK4A}$ nuclear immunoreactivity. *F*, 129 hormone-naïve patients categorised by $p16^{INK4A}$ strata in areas of HGPIN (log rank, $P = 0.013$). □, ⩽ 1% $p16^{INK4A}$ nuclear immunoreactivity;○, >1% $p16^{INK4A}$ nuclear immunoreactivity. From Henshall and colleagues [54] with permission from the American Association for Cancer Research.

expression as the result of pRb loss is an attractive hypothesis to explain this. However, if this is correct then one might expect pRb loss to be prognostic by itself (see above) and the apparent loss of a defined relationship between $p16^{INK4A}$ and CDK4 between benign and malignant prostate tissue is left unexplained. In a prostate cancer xenograft model, examining cell cycle changes with androgen withdrawal and development of androgen independence, $p16^{INK4A}$ expression increased progressively after androgen withdrawal and plateaued, but was then unchanged with development of androgen-independence. These fluctuations

occurred in the presence of easily detectable pRb expression, suggesting that the development of resistance to the inhibitory effect of $p16^{INK4A}$ may occur independent of pRb status.

$p16^{INK4A}$ overexpression appears to be prognostic in prostate cancer treated with RP, but this needs confirmation in other cohorts and, ideally, in patients treated with modalities other than surgery. The status of other components of the $p16^{INK4A}$/Rb pathway, particularly pRb expression, need to be evaluated concurrently so that the underlying mechanisms that result in $p16^{INK4A}$ overexpression being prognostic can be understood.

3.4. $p21^{WAF1/CIP1}$

The use of $p21^{WAF1/CIP1}$ as prognostic biomarker in a range of tumours has yielded conflicting results, with studies reporting that reduced or increased expression of $p21^{WAF1/CIP1}$ can be adversely prognostic within the same tumour type, breast cancer being the prime example [75]. The causes of variation of $p21^{WAF1/CIP1}$ expression in tumours are still to be fully elucidated. $p21^{WAF1/CIP1}$ is rarely mutated in human cancers, although several prostate cancers containing point mutations have been reported [76]. Reduced $p21^{WAF1/CIP1}$ expression might be expected in the presence of p53 dysfunction, given the original proposal that it was a primary regulator by which p53 asserts cell cycle inhibition ($p21^{WAF1/CIP1}$/p53 concordance). However, it has subsequently become clear that $p21^{WAF1/CIP1}$ is also regulated by other factors and can exert inhibitory effects on the cell cycle and apoptosis independent of p53 effect in both settings of development and cancer [32,77,78]. Several investigators have demonstrated that $p21^{WAF1/CIP1}$ expression does not correlate with p53 status in a variety of cancer types [75,79]. The reasons for this are complex, but include alternative regulation of $p21^{WAF1/CIP1}$ by other molecules, particularly those involved in cell cycle regulation, altered degradation or p53 mutation that alters its effect on apoptosis but not on $p21^{WAF1/CIP1}$ and the cell cycle [77,80]. One further possible explanation in prostate cancer is that androgens may directly stimulate $p21^{WAF1/CIP1}$ transcription [81]. It is likely that $p21^{WAF1/CIP1}$ expression is the result of an epigenetic response to several cellular regulators and that either reduced or increased expression could be prognostic in certain environments. Generally, clinical studies suggest that increased $p21^{WAF1/CIP1}$ expression is adversely prognostic in patients treated with RP (Table 4). Recent work suggests that $p21^{WAF1/CIP1}$ overexpression may predict resistance to radiation therapy for local recurrence after RP [82] and with the development of hormone-refractory prostate cancer [83].

Hence, $p21^{WAF1/CIP1}$ overexpression is prognostic in localised prostate cancer independent of p53 status. Its inter-relationship with other cell cycle regulators and various p53 alterations in predicting outcome requires further evaluation.

3.5. $p27^{Kip1}$

$p27^{Kip1}$ is encoded by a gene at 12p12-12p13.1 [84]. $p27^{Kip1}$ null mice are characterised by diffuse hyperplasia or hypertrophy of glandular organs including the prostate [85,86]. Despite the fact that no or reduced $p27^{Kip1}$ expression is adversely prognostic in several cancers [59,75,87–93], mutation is a rare event in early human tumours [84,94,95]. An interesting recent finding is that homozygous deletion at 12p12-12p13.1 was present in 47% of patients dying of metastatic prostate cancer, suggesting that this genetic change might be a significant late event in the progression of prostate cancer [96]. Reduction in $p27^{Kip1}$ expression in localised tumours comes about through post-transcriptional regulation, predominantly through a proteasome-associated ubiquitin-mediated degradation mechanism [86,92,97–99]. In transition from benign prostate to prostate cancer, as well as other tumours, this degradation mechanism is turned on without compensatory increase in protein production [86,100]. There are also examples of $p27^{Kip1}$ overexpression being adversely prognostic [101], presumably as an epigenetic regulatory response to other cell cycle molecular aberrations [90]. $p27^{Kip1}$ function and expression is complementary to that of cyclin E in some systems, while its inhibitory effect may also be abrogated by c-Myc overexpression [102–105]. Studies of *in vitro* and xenograft systems show that the expression and cell cycle inhibitory effect of $p27^{Kip1}$ are reduced by androgen stimulation [106–108]. Castration or androgen depletion results in an increase in $p27^{Kip1}$ expression with concurrent cell cycle arrest. Re-introduction of androgen or the development of androgen independence results in reduced $p27^{Kip1}$ expression to levels lower than before castration. The implication from this work is that $p27^{Kip1}$ plays an important role in maintenance of cell cycle arrest in androgen-dependent tumours and that reduced expression, particularly in the setting of androgen deprivation, may indicate the presence of androgen-independent prostate cancer cells [107,108].

Several studies have examined the relationship between $p27^{Kip1}$ expression and clinical outcome and found that low or undetectable $p27^{Kip1}$ protein is associated with increased tumour grade and stage and remains an independent predictor of treatment failure after prostatectomy defined by PSA and/or clinical recurrence (Table 4) [86,109–115]. $p27^{Kip1}$ expression appears not to correlate with pre-operative serum PSA [111]. Different studies use different percentages of cancer nuclei staining, ranging from 10% to 50%, to dichotomise

cohorts. In one of these studies, neoadjuvant hormonal therapy (NHT) was administered pre-operatively to 24 of 113 patients in a variable manner [110]. Tumours from patients who received NHT tended to express higher levels of $p27^{Kip1}$ than did those from untreated patients. Patients whose tumour $p27^{Kip1}$ remained low after NHT had a shorter relapse-free survival. Together this suggests that $p27^{Kip1}$ is a key molecule in prostate cancer cell response to androgen.

Hence, reduced $p27^{Kip1}$ expression predicts a shorter disease-free survival in patients with localised prostate cancer in most reported series. The predictive importance of $p27^{Kip1}$ in patients treated with NHT requires evaluation in other cohorts. The inter-relation of $p27^{Kip1}$ with other components of the cell cycle and the predictive potential of $p27^{Kip1}$ in prostate biopsies and with treatment modalities apart from surgery should also undergo further investigation.

3.6. c-Myc

Chromosome 8 is often subject to alteration in prostate cancer, with loss on 8p and gain on 8q being common (Table 2) [116–118]. While a series of potential tumour suppressor genes are postulated in the region commonly lost at 8q21-22, amplification at the 8q24 locus which includes *C-MYC* has attracted more attention because of its prognostic use in prostate cancer [119]. A series of investigators report that *C-MYC* amplification is present in up to 50% of high-grade prostatic intraepithelial neoplasia (HGPIN) and 73% of primary prostate cancer [120–122] and that *C-MYC* amplification increases with transition through prostatic intra-epithelial neoplasia (PIN) to localised prostate cancer to metastases [122,123] and with increasing Gleason score [124,125]. In a recent publication on 144 patients with high-grade, locally advanced (pT3) prostate cancer, Sato and colleagues [119] found that increased copy number for *C-MYC* using fluorescence in situ hybridisation (FISH) strongly predicted systemic progression and patient death. In addition, aberrations elsewhere on chromosome 8 appear to occur in sequence with *C-MYC* amplification and influence outcome in a cumulative manner that requires further study.

C-MYC amplification is a feature of increasing grade and stage in prostate cancer and predicts adverse outcome in locally advanced disease. The technical demands of FISH make it difficult to apply in routine practice. While some studies report a good correlation between amplification on FISH and c-Myc overexpression by IHC, this has not been universal, and furthermore c-Myc overexpression detected by IHC has not been demonstrated to be of prognostic significance. *C-MYC* is an important oncogene in prostate cancer, but more research is required to determine its relationship with other biomarkers and its prognostic role in subsets of prostate cancer patients.

4. Apoptosis (programmed cell death)

The major apoptotic regulators, p53 [126] and Bcl-2 [127] both demonstrate abnormal function and expression as prostate cancer progresses and are mechanistically implicated in hormone resistance [128–133]. Following therapy with androgen ablation, p53 and Bcl-2 expression as well as the ApI increases in a large proportion of cases [134]. Failure of apoptotic response as measured by the ApI correlates with relapse [134]. In addition, the mean increase in Bcl-2 expression is greater in cases that do not respond to hormone therapy or progress early after its commencement [134]. *In vitro* and animal tumour xenograft experiments demonstrate resistance to spontaneous as well as androgen deprivation-, radiation- or chemotherapy-induced apoptosis mediated, at least in part, by Bcl-2 overexpression [129,135].

4.1. p53

p53 functions by regulating the transcription of genes involved in G_1-phase growth arrest of cells in response to DNA damage. p53 also has roles in the regulation of the spindle checkpoint, centrosome homeostasis and G_2–M phase transition [136]. p53 regulates apoptosis [126,136,137] and tumour angiogenesis in benign and malignant cells [126,138–141]. Hence, the effects of p53 related to cancer can be summarised into three processes: cell cycle regulation, apoptosis and angiogenesis/metastasis.

Nuclear accumulation of p53 detected by IHC typically indicates the presence of *iip53* gene mutations [142,143], although the correlation between nuclear accumulation of p53 and the presence of *p53* gene mutation can vary [144]. Lack of p53 accumulation may occur in the presence of *p53* mutations, particularly non-sense mutations with truncated *p53*, single-base mutations not causing any change in the amino acid sequence and mutations outside of exons 5–8 [145,146]. Mutations that limit the ability of p53 to interact with regulatory proteins such as Mdm-2 may cause p53 nuclear accumulation, but such mechanisms are dependent on other cellular factors, such as DNA integrity [147]. Regardless of the mechanisms involved, nuclear accumulation of p53 is a prognostic indicator in several human cancers, including breast [139,148,149], lung [150] and colorectal carcinoma [151].

The value of p53 nuclear accumulation as a prognostic factor in localised prostate cancer has been debated. A number of studies have shown that p53 nuclear accumulation detected by IHC is prognostic at a variety of

dichotomising cut-off points, based on number of p53-positive nuclei. These studies either describe a poor prognosis group of patients with $\geqslant 20\%$ p53-positive nuclei [79,128,152,153] or a group of patients with lower percentages of positive cells in a heterogeneous, focal staining pattern where either the presence of any nuclear accumulation or the presence of clusters of cells showing nuclear accumulation is adversely prognostic [130,154,155]. However, other studies comparing p53 nuclear accumulation with assessment of *p53* gene mutations have failed to provide conclusive evidence for the importance of p53 in localised prostate cancer or a strong correlation between nuclear accumulation and *p53* gene mutation [156–159]. In studying other cancers, several authors have suggested that assessment of *p53* gene mutation and p53 expression in combination may more accurately define prognostically important p53 dysfunction [144,160,161].

Comparison of prostate cancer metastases with primary prostate cancers in the same patients suggest that foci with *p53* mutations are clonally expanded in metastases [132,162–164], perhaps explaining the high frequency of IHC positivity and presence of gene mutations in hormone refractory and metastatic prostate cancer [23,157–159,165–168]. Two studies have demonstrated significant heterogeneity in the distribution of *p53* mutations between and within foci of carcinoma in the same prostate [169,170]. Other studies document heterogeneity for other genes and suggest that clones responsible for metastases do not always originate from within the dominant tumour focus [122,171,172]. Recent work demonstrates the focal presence of *p53* mutations within areas of p53 protein accumulation detected by IHC [173]. The likelihood exists that in localised prostate cancer, p53 overexpression and mutation, as well as other genetic aberrations, may be limited to subgroups of prognostically important malignant cells. These studies add to others that demonstrate increased p53 nuclear accumulation in metastatic, recurrent and/or androgen-insensitive prostate cancer compared with clinically localised disease [23,157–159,166–168]. Borre and colleagues [174] reported on a population of patients observed with no treatment after prostate diagnosis and found p53 nuclear accumulation to be predictive of prostate cancer related death. In work carried out at the Garvan Institute, Quinn and colleagues demonstrated the increasingly adverse prognostic effect of an increased percentage of cell with p53 nuclear accumulation that was independent of PSA, Gleason score and pathological stage [133] (Fig. 3). Interestingly, at low levels of p53 expression, the presence of clusters of 12 or more p53 positive cells was adversely prognostic (Fig. 2(a)) [133]. Taken together, these studies suggest that prostate tumour cells harbouring p53 mutations and perhaps other genetic aberrations are clonally expanded in metastases.

There are more than 100 studies reporting series of patients with prostate cancer evaluated for p53 nuclear accumulation. No attempt will be made to recapitulate this expansive literature here. Essentially this literature demonstrates increasing p53 expression with increasing grade and stage with a prognostic effect that may or may not be independent of these two variables. However, several studies examine the issue of clinical use of p53 in pre-treatment biopsy material in particular therapeutic settings that deserve further scrutiny and are summarised in Table 5. p53 is a prognostic marker in prostate cancer, however, because of the heterogeneity of aberration in localised disease and a surfeit of therapeutic agents with potential to abrogate its effect, it has practical and clinical limitations.

4.2. Bcl-2

The *bcl-2* gene was initially identified as the proto-oncogene translocated to the immunoglobulin (Ig) heavy-chain locus in follicular B-cell lymphoma. It is the prototype of a novel class of oncogenes that inhibit apoptosis or programmed cell death [175,176]. Bcl-2 is part of an expanding family of apoptosis-regulatory molecules, which may act as either death antagonists (Bcl-2, Bcl-xL and Mcl-1) or death agonists (Bax, Bak, Bcl-xS, Bad and Bid). The selective and competitive dimerisation between pairs of antagonists and agonists determines how a cell will respond to a given signal [127].

Within the prostate, Bcl-2 expression is commonly seen in the basal layer of benign glands, PIN and some cancer, whereas expression in secretory layer epithelial cells is abnormal. Several studies [129–131,177–180] demonstrate that increased expression of Bcl-2 in prostate cancer confers androgen resistance, particularly in advanced disease, and may facilitate progression to androgen independence. Stattin and colleagues studied Bcl-2 expression in two similar sets of patients treated with castration for locally advanced prostate cancer and found that Bcl-2 increased in both responders and non-responders but that the increase was far greater in responders and correlated with ApI [134]. Recent work suggests that Bcl-2 overexpression has a role in resistance to radiotherapy in prostate cancer [181]. A number of studies have proposed that increased Bcl-2 expression is adversely prognostic in localised prostate cancer and a selection of these is presented in Table 5.

These studies suggest that Bcl-2 expression increases with grade and stage. For this reason, Bcl-2 overexpression may be useful prognostically in relatively more advanced tumours, such as those selected to have radiotherapy or hormonal therapy rather than RP. This is one potential reason that Bcl-2 expression on biopsy may be independently prognostic in radiotherapy cohorts and not those treated with RP. Another is that

Table 5
Selected studies on the apoptotic markers, p53 and Bcl-2, and outcome in prostate cancer

Molecule	Treatment	Cohort size	Stage	Dichotomising level for marker expression	Effect on prostate cancer outcome	Special notes	References
p53							
	Observation – cohort	221	Unrestricted at presentation	>50% nuclear accumulation – biopsy	Independently prognostic – predicted overall survival	Hormonal therapy at clinical progression	[174,405,406]
	External beam radiation therapy with hormone therapy – RTOG 8610	129	Clinically localised, T1-3, N0	>20% nuclear accumulation – biopsy	Independently prognostic – distant metastases	p53 status predicted for patient given radiotherapy + hormones but not those given radiotherapy alone	[152]
	RP	263	Clinically localised, T1-3,N0-1	Variety: Cluster of 12 cells, >5, 20% nuclear accumulation – prostatectomy	Independently prognostic – PSA recurrence	>20% nuclear accumulation predicted prostate cancer related death	[3,4,133]
	RP	175	Clinically localised, T1-3,N0-1	>0% nuclear accumulation – prostatectomy	Independently prognostic – PSA recurrence	Also found Bcl2 overexpression prognostic	[130,154]
	RP	129	Clinically localised, T1-3,N0-1	>0% nuclear accumulation – biopsy	Not prognostic in biopsy		[130,154,407]
	RP	76	Clinically localised, T1-3,N0-1	>0% nuclear accumulation – biopsy and prostatectomy	Prognostic in both – PSA recurrence		[302]
Bcl-2							
	RP	175	Clinically localised, T1-3,N0-1	>0% nuclear accumulation – prostatectomy	Independently prognostic – PSA recurrence	Also found p53 overexpression prognostic	[130,154]
	RP	129	Clinically localised, T1-3,N0-1	>0% nuclear accumulation – biopsy	Not prognostic in biopsy		[130,154,407]
	RP	76	Clinically localised, T1-3,N0-1	>0% nuclear accumulation – biopsy and prostatectomy	Prognostic in prostatectomy but not in biopsy – PSA recurrence		[302]
	External beam radiation	52	Clinically localised	>0% nuclear accumulation – biopsy	Prognostic		[408]

RP, radical prostatectomy; RTOG, Radiation Therapy Oncology Group; PSA, prostate-specific antigen.

patients with Bcl-2 overexpression do better with radiotherapy because the cells are more sensitive to its effects. A prospective trial of radiotherapy with cases stratified for normal against Bcl-2 overexpression in prostate cancer on biopsy and matched for clinical stage and Gleason score could test this hypothesis in a clinical setting.

5. Androgen receptor signalling

The expression of the steroid hormone receptors is prognostic in a number of hormone-dependent tumours. The best example is the oestrogen receptor (ER) in breast cancer, where loss of expression of ER predicts a more aggressive disease course independent of treatment given and resistance to hormonal therapies such as tamoxifen. While ER expression is altered with prostate cancer progression [182], AR expression represents a more obvious potential marker of prognosis and hormonal responsiveness in prostate cancer. Early studies of AR expression provided conflicting results and only served to demonstrate that heterogeneity increases markedly with progression from benign through PIN to localised prostate cancer and metastases [183–190]. In examining tumour material from 30 patients with prostate cancer, Tilley and colleagues used anti-peptide antibodies to the amino- and carboxy-termini of the AR and demonstrated differential expression as the disease progressed from early stage to bone metastases [191]. More advanced disease had increased expression of amino-terminus epitopes. The prognostic significance of carboxy-terminus epitope expression remains to be determined, but it has been suggested that differential expression between amino- and carboxy-epitopes may correlate with AR mutation and/or amplification [192]. Recent studies using commercially available antibodies directed at either the amino-terminus or the whole AR molecule [415] suggest that AR overexpression is a feature of progression, recurrence, lymph node (LN) metastases and/or anti-androgen resistance in human prostate cancer [191,193–199]. A recent study failed to find an association between AR expression in the primary prostate cancer and outcome, but did find that AR expression in >70% of LN metastases predicted for a poorer cancer-specific survival in the subset of patients with LN involvement after controlling for Gleason score and pre-operative PSA in multivariate analysis [199,200].

The relationship between AR expression and mutation or amplification of the AR gene is poorly understood. However, several studies suggest that overexpression may correlate with mutation and/or amplification and with androgen resistance [201–203]. High-level AR amplification has been described in more than 30% of prostate cancers recurring after androgen ablation [202,204]. A recent report links AR overexpression in prostate cancer epithelial cells in combination with reduced AR expression in the surrounding stromal cells with increasing tumour grade [197]. Further work in our laboratory at the Garvan Institute demonstrates that the combination of high AR expression in the epithelium and reduced expression in the adjacent stroma is adversely prognostic. This suggests the presence of aberrant stromal signalling through a paracrine mechanism [205] and provides supporting evidence for paracrine stromal–epithelial regulation of AR expression as prostate cancer progresses [203]. It is possible that overexpression of AR results in part from lack of response to homeostatic degradation mechanisms [206] in cells with a mutated or amplified AR gene, while adjacent stromal cells have normal AR which is down-regulated. In addition, a number of growth factors have been implicated in prostate cancer stromal–epithelial interaction including TGFß [207,208], yet to be identified TGF analogues [209], the fibroblast growth factor (FGF) family including keratinocyte growth factor (KGF, FGF7) and FGF10 [210–212] and a variety of cytokines including IL-6 [213]. TGFß is progressively overexpressed in epithelial cells with prostate cancer progression, with corresponding loss of TGFß cell cycle inhibition [208]. In prostate stromal cell models, TGFß blocks androgen-induced proliferation and results in re-distribution of AR from the nucleus to the cytoplasm [214,215]. Recent work demonstrates that increased preoperative serum TGFß levels predict relapse after RP [216].

A complex picture has emerged with the evaluation of recurrent or metastatic prostate cancer for AR mutation and amplification. In evaluating material from 10 bone marrow metastases from clinically hormone-independent prostate cancers, Taplin and colleagues [217] found that AR was highly expressed. Fifty percent (5/10) of these prostate cancers contained AR mutations associated with promiscuous receptor stimulation by a variety of hormones, including progesterone, adrenal androgens and oestrogen, as well as paradoxical stimulation by the anti-androgen, hydroxy-flutamide. Subsequent studies by the same group and others [193,218–222] demonstrated a high propensity for mutation at a single site (codon 877) in patients treated with flutamide and for these mutations to predict flutamide withdrawal response in androgen-insensitive disease. It has since become evident that biclutamide therapy is associated with AR mutation and amplification in metastases, suggesting that the effect is at least class-specific to non-steroidal anti-androgens [223]. Tilley and colleagues [224] identified a series of different AR point mutations in 44% of 25 hormonally naïve patients with the incidence of mutation increasing with disease stage. Individual AR mutants had differential binding affinity for different hormones and different downstream effects [224–230]. It has suggested that pathways downstream of the AR can be stimulated by aberrant activation of the erbB2

(HER-2/neu receptor tyrosine kinase) pathway [231]. The cellular model used in this system also suggests that activation of the AR pathway can be synergistically mediated through effect of both erbB2 and AR [231,232]. There is experimental and human prostate cancer tissue evidence for retinoblastoma protein, c-Myc, interleukin-4 and 6 and p53 regulation of AR expression [39,233–237].

Hence, it is possible that in selected prostate cancer cells, AR may be amplified, overexpressed through epigenetic regulation and/or contain mutations that allow stimulation by a range of hormones and anti-androgens [238]. It is likely that therapy, particularly with non-steroidal anti-androgens, may provide selective pressure that leads to preferential expression of cells with AR mutations or amplification. In addition, autocrine or paracrine mechanisms may, in lieu of, or in concert with AR, activate pathways to produce downstream AR responses [238]. Determining which mechanisms are active at various stages of prostate cancer progression and how they might interact with other molecular markers of prostate cancer virulence is clearly important.

6. 5-α reductase

The expression of 5-α reductase is increased in high-grade and androgen-insensitive prostate cancer [239]. There may also be a cancer progression related shift in localisation of 5-α reductase from the nucleus to the cytoplasm [239]. The prognostic implications of these observed changes require further evaluation.

7. Signal transduction

7.1. Epidermal growth factor receptor family

Aberrant expression of the epidermal growth factor receptor (EGFr) family is common in prostate cancer [240–242], although the extent and prevalence of this varies depending upon techniques used to demonstrate it and the population studied. Convergent signalling between androgen-regulated processes and the pathway by which signals are transduced from cell surface receptor through Raf-1, MEK, MAP kinase and p27KIP1 have been mechanistically delineated in cell culture models, although the clinical relevance of these findings is still unclear [243].

HER-2/neu is a candidate marker for predicting prostate cancer progression. Varying rates of HER-2/neu overexpression ranging from 9% to 64% of prostate cancers has been reported, depending on the investigative method employed and the specificity of reagents used [244–250]. Overexpression of HER-2/neu correlated with increasing grade and increasing stage in separate series [240,244]. Fox and colleagues [246] found that overexpression of HER-2/neu by IHC predicted outcome in T1A prostate cancers. HER-2/neu amplification assessed by FISH was associated with disease recurrence in a series of 106 primary tumours [247]. Within the California Cancer Consortium, patients were screened prospectively for shed serum Her2 antigen, IHC and FISH but demonstrated a very low rate of abnormal expression and no response to trastuzumab, a therapeutic monoclonal antibody directed at Her2/neu [251]. Activation specific antibodies for HER-2/neu may permit the delineation of a group of prostate cancer patients with functional overactivity as distinct from overexpression. Other members of the erbB family including EGF-R, c-erbB3 and c-erbB4 may warrant evaluation as prognostic markers [252–254].

7.2. Ras

C-RAS was one of the first oncogenes identified and point mutations that correlate with increased ex vivo activity were an important part of models of prostate cancer in rodents [207,255,256]. Early studies in human tumours showed that activated Ras was increasingly evident as the disease progressed into the metastases [257]. However, detection of activating mutations of *C-RAS* in localised tumours proved variable and it soon became clear that their measurement was not likely to be of important prognostic significance [258–260]. Studies using antibodies to Ras peptide sequence have failed to demonstrate a relationship with clinico-pathological or outcome parameters [261]. Most of the studies undertaken on Ras have occurred in North American Caucasian populations. There is significant racial variation in *C-RAS* point mutation type and frequency and therefore Ras activity and expression may warrant further investigation as a prognostic marker in other racial groups [262,263].

7.3. Phosphoinositide 3-kinase/Akt pathway

The phosphoinositide 3-kinase/Akt pathway is an important signal transduction pathway in many cell types and influences cycle kinetics via $p27^{Kip1}$ regulation [264–267]. Within this pathway there are several molecules that demonstrate altered expression in a variety of cancers [268]. In murine prostate carcinogenesis models, prostate-specific deletion of PTEN resulted in metastatic prostate cancer, while AKT activation saw the development of PIN [269,270]. PTEN is a tumour suppressor phosphatase that is commonly altered in lethal metastatic prostate cancer [164,271–275]. *In vitro* work shows that reconstitution of PTEN suppresses AR transcription and increases sensitivity to cytotoxic drugs [276,277]. This may have

clinical relevance, since the PI3K/Akt and MAP kinase pathways become hyperactive with development of androgen independence in paired tumour samples [278]. While loss of PTEN expression correlates with increased Gleason score and increased pathological stage in patients with clinically localised prostate cancer, evidence of an effect on outcome has been lacking [275]. Similarly, increased Akt expression is reported to correlate with increased Gleason score and pre-therapy PSA concentration >10 ng/ml in localised prostate cancer, but a link to outcome has not been delineated [279,280].

8. Cellular adhesion/cohesion

8.1. E-cadherin and related molecules

E-cadherin is involved the regulation of cell–cell adhesion and cell morphology [281,282]. Functionally, cadherins form a complex with other molecules of importance in this process, particularly catenins [283,284]. Reduced expression of one or more components of this complex has generally been associated with a more aggressive cancer phenotype, as measured by a number of parameters, as well as a poorer outcome in a number of cancers, including prostate cancer [285–289]. Down-regulation of E-cadherin expression in localised prostate cancer is associated with increased expression of other cadherin family members, particularly N-cadherin [290]. It has been suggested that while E-cadherin promotes epithelial cell–epithelial cell adhesion, N-cadherin promotes epithelial cell–stromal cell adhesion [291]. In an apparent paradox, E-cadherin is overexpressed in metastases [292,293]. This suggests that E-cadherin expression is transiently turned off through an epigenetic mechanism during invasion and diapedesis into vessel walls, only to be reactivated at the site of established metastases [292]. Other cadherins may play a role in this switching. 'Re-expression' of E-cadherin has also recently been described in the transition from primary to metastases in breast cancer [294]. Recent work has focused on interaction of the cadherins with other molecules that modulate their function by truncation of the cadherin protein or stoichiometrically [295–300]. The impact of these modulators on clinical prognostication and therapeutics requires further delineation.

There are several studies evaluating E-cadherin expression and prostate cancer outcome, as summarised in Table 6. Overall, they suggest a significant prognostic effect for E-cadherin expression in prostate cancer. Clinical use of E-cadherin expression is limited by heterogeneous expression in prostate cancer, thus biopsy results may not be predictive [301,302]. Hence, E-cadherin and related molecules have potential as prognostic markers in prostate cancer, but require further testing in large cohorts to determine whether they are independent markers of outcome and if the effects are related to specific subgroups.

The Wnt signalling pathway mediates a variety of cellular functions, including cell polarity, tissue patterning, control of cellular proliferation and development of neoplasia [303–305]. This pathway is initially activated by a Wnt ligand binding to a Frizzled receptor, which subsequently transduces a signal through activation of β-catenin [305]. Although expression of Wnt ligands [306,307], Frizzled receptors [307,308] and ß-catenin [309–311] in prostate cancer has been established for many years, recent studies have demonstrated that Wnt 1 [311], nuclear ß-catenin [312] and the Wnt-pathway inhibitor, secreted frizzled-related protein 4 (sFRP4) [313], have an association with prostate cancer outcome. Increased Wnt-1 expression correlates with increased Gleason score and serum PSA levels, which is consistent with its role as an oncogene [311]. Conversely, increased expression of sFRP4 in a membranous pattern of immunostaining in >20% of malignant epithelial cells independently predicts for a longer biochemical relapse-free survival in patients with localised prostate cancer ($P = 0.02$) [313]. Interestingly, loss of ß-catenin expression in the nucleus of malignant epithelial cells is associated with both prostate cancer progression and an increased risk of relapse in localised prostate cancer in particular in the low-risk subgroup of patients with pre-operative PSA levels < 10 ng/ml [312]. *In vitro* studies suggest that ß-catenin signalling in the nucleus can promote or repress tumour growth and development depending on the co-factors present [309,314–316] and it may be the balance of these effects towards tumour repression in prostate cancer that accounts for higher levels of nuclear ß-catenin predicting for a better prognosis.

Altered expression of molecules in the prostatic stroma, including chondroitin sulphate [317–319] and hevin [320] as well as cell surface markers such as CD44 [247,321] also have prognostic impact and represent potential therapeutic targets.

9. Angiogenesis

Neoangiogenesis is essential for the growth and metastatic propagation of cancer. Increased microvessel formation is a feature of many cancers including prostate cancer where quantification of microvessel density correlates with disease stage and outcome [139,141,322–326]. Aberrant blood vessel formation is associated with anomalies in pathways involved in apoptosis, AR signalling, signal transduction, cytokine function and

Table 6
Selected studies of cellular adhesion and cohesion molecules

Molecule	Treatment	Cohort size	Stage	Dichotomising level for marker expression	Effect on prostate cancer outcome	Special notes	References
Reduced E-cadherin					Summary: Prognostic		
	RP/TURP	89 (42/47)	Clinically localised/ locally advanced, T1-4,N0-1	Nil expression or aberrant location (non-basal layer expression)	Prognostic		[370]
	RP	67	T1-4,N0-1	Low expression	Prognostic for disease-free survival		[409]
	RP	72	Clinically localised, T1-3,N0-1	Aberrant expression	Prognostic for disease-free survival – PSA recurrence	Biopsy expression non-prognostic	[302]
	RP	56	T1-4,N0-1	Low expression	Prognostic for early development of clinical metastases		[410]
	TURP	99	T1-4,N0-1, M1	Low expression	Independently prognostic for overall survival relative to Gleason score but not presence of metastases		[286]
Increased chondroitin sulphate	RP	157	T1-4,N0-1	Absorbance cut point above 7.0	Independently prognostic for PSA recurrence	May be most useful in patients with PSA < 10 ng/ml	[317–319]

RP, radical prostatectomy; PSA, prostate-specific antigen; TURP, transurethral resection of prostate.

cellular adhesion [140,234,327–330]. Blood vessel formation is regulated by molecules involved in adhesion as well as vascular endothelial growth factor (VEGF) [331], nitric oxide and cyclo-oxygenases. VEGF is crucial for the development of tumour masses exceeding a diameter of 3–5 mm [332].

Pre-clinical data with prostate cancer cell lines demonstrate that VEGF is a potentially important factor in stimulating cell proliferation as well as angiogenesis and lymphangiogenesis [329]. In experimental prostate cancer models, VEGF expression is upregulated in prostate and prostate cancer tissue by androgens and castration results in an initial fall in VEGF [333–335]. The expression and effect of VEGF is regulated by a series of heterogeneous molecules. These include neuropilin, activator protein 2-α-angioproteins, ephrins and interleukin 6 and 8 [336–340].

VEGF is highly expressed in most prostate cancers [322,326,341]. The distribution of VEGF within prostate cancers is interesting. As expected, there are significant levels in endothelial cells and in the cytoplasm of cancer cells, with parallel increase with Gleason grade [342]. Neuroendocrine cells are highly expressive of VEGF isoform A and, in contradistinction to endothelial and adenocarcinoma cells where levels are androgen-dependent, there is no fall in expression with androgen blockade [326,342–345]. This finding may have therapeutic implications for hormone-resistant disease and VEGF targeting. Higher VEGF tissue expression predicts biochemical PSA relapse following prostatectomy [327] and death from prostate cancer in a cohort that underwent observation for clinically localised disease [326] and two other cohorts with HRPC [346,347]. In patients undergoing RP, elevated preoperative serum or urine VEGF levels are predictive of earlier disease progression [348,349]. Serum VEGF falls after prostatectomy [350]. Patients with metastatic prostate cancer have serum VEGF concentrations significantly higher than normal populations [351,352]. There are at least four isoforms of VEGF (A, B, C and D) each with different roles and receptor affinities, but without clear differential prognostic or predictive ability at this time [353].

VEGF receptor expression occurs diffusely through prostate carcinoma [354]. Each of the receptors have different physiological roles [353]. VEGFR1 (Flt-1) promotes vessel sprouting and branching while inhibiting tubular elongation possibly through release of soluble component that negatively modulates VEGF and VEGFR2 (Flk-1/KDR) [355,356]. VEGFR2 promotes tubular elongation of blood vessels, while VEGFR3 (Flt-4) is directed at lymphangiogenesis and possibly lymph node metastasis [357]. VEGFR2 signalling is responsible for increased prostate cancer cellular proliferation as well as neoangiogenesis [358,359]. In prostate cancer progressive disease is associated with decreased VEGFR1 and increased VEGFR2 [360]. Recent work suggests that VEGF and VEGFR2 may have a role in the development of osteoblastic bone metastases that are characteristic of advanced prostate cancer [361]. The mechanism postulated for this involves preferential expression of integrins on the cell surface. Higher VEGF receptor 3 expression predicts early tumour progression after RP [362]. Increased VEGFR3 expression in lymphatic endothelial cells predicts increasing disease stage and particularly lymph node involvement at RP [363].

Hence, VEGF expression is prognostic in prostate cancer, while varied expression of VEGFR2 and VEGFR3 may have respective roles in bone and lymph node metastases.

10. Gene expression profiling to delineate markers of outcome

A contemporary approach to discover new genes of prognostic significance is to utilise microarray analysis to define gene expression profiles that co-segregate with poor clinical outcome [10,364–367]. The most advanced published data on the use of such an assay is currently in breast cancer, where the Netherlands Cancer Institute and Antoni van Leewenhoek Hospital have pioneered the use of microarray profile analysis based on 70 genes, in conjunction with conventional prognostic tests to determine which women should receive adjuvant treatment after surgery [365,366]. The use of a microarray-based prognostic tool in the treatment of prostate cancer is under development. Three prostate cancer gene expression datasets have utilised primary prostate cancers with outcome data in an attempt to define gene expression profiles associated with prostate cancer recurrence [10,367,368] (Table 7a). While a comprehensive meta-analysis of these data is still to be performed, commonalities do exist. Lapointe and colleagues reported that while there was no overlap between the 23 genes associated with early recurrence in their cohort and those identified by Singh and colleagues, their set of genes predicted recurrence for patients included in the latter study. Similarly, none of the probe-sets identified by Henshall and colleagues overlapped with the probe-sets selected by Singh and colleagues. However, a potential functional link was noted between the transient receptor potential (TRP) channel trp-p8 and calnexin, both predictors of outcome in the Henshall and colleagues study, and chromogranin A and inositol triphosphate receptor identified by Singh and colleagues, because TRP ion channels are linked to the phosphatidylinositol signal transduction pathway. Importantly, the data from all three studies provide strong evidence for a gene expression profile of poor prognosis in localised prostate cancer. The critical next steps in developing a clinically useful gene expression panel for prostate cancer is to assure the fidelity of sample preparation (e.g.,

Table 7a
Selected studies of expression profiling of prostate cancers to delineate markers of poor prognosis

Cohort with recurrence data	Cohort follow-up	Array platform	Definition of recurrence	Key findings	References
$n = 21$ 8 recurrences	13 relapse-free patients >4 years post-RP	U95Av2 Affymetrix oligonucleotide array (~10,000 genes)	Two successive PSA > 0.2 ng/ml	No single gene was statistically associated with recurrence; a 5 gene outcome prediction model was used to predict recurrence[a]	[367]
$n = 72$ 17 recurrences	28.25 months median follow-up	Customised Affymetrix oligonucleotide array (~46,000 unique sequences)	Two successive PSA > 0.3 ng/ml ± clinical recurrence	~200 probe-sets showed strong correlation with relapse with additional predictive value relative to preoperative serum PSA	[10]
$n = 29$ 7 recurrences	11.5 months median relapse-free survival	cDNA array (26,260 genes)	PSA > 0.07 ng/ml or occurrence of clinical metastasis	4 genes positively, 19 genes negatively associated with early recurrence; validated MUC1 and AZGP1 as prognostic	[368]

PSA, prostate-specific antigen.
[a] The top 5 genes used in over half of the models included chromogranin A, platelet-derived growth factor receptor ß, HOXC6, inositol triphosphate receptor 3 (IPTR3) and sialyltransferase-1.

Table 7b
Selected studies of validated prognostic markers identified by gene expression profiling of prostate cancers

Molecule	Treatment	Cohort size	Effect on prostate cancer outcome	Special notes	References
Hepsin	RP	78	Absent or low expression is prognostic		[411,412]
PIM1	RP	78	Decreased expression is prognostic		[412]
EZH2	RP	64	Moderate to strong expression is prognostic	Increased expression in metastatic prostate cancer[a]	[300,413]
MTA1	RP	108	Decreased expression is prognostic	Increased expression in metastatic prostate cancer[a]	[414]
MUC1	RP	225	Increased expression is prognostic		[368]
AZGP1	RP	225	Decreased expression is prognostic		[368]

RP, radical prostatectomy.
[a] Relative to localised prostate cancer and benign prostate [413,414].

laser-captured populations of malignant epithelial cells), adapt a widely available array platform, and translate the approach for application to fixed tissues to enable analysis of larger cohorts of patients with longer follow-up.

An increasing number of potential prognostic markers identified by gene expression profiling are being validated using IHC on tissue microarrays comprised of large cohorts of patients treated for localised disease with RP (Table 7b). The clinical use of these molecules in identifying patients with aggressive prostate cancer will ultimately need to be analysed for their ability to improve pre-operative prediction of prostate cancer recurrence [5].

11. Conclusion

A limited number of molecular markers in prostate cancer tissue are of clinical use in predicting outcome or response to therapy. Current markers with potential include p53, Bcl-2, $p16^{INK4A}$, $p27^{Kip1}$, c-Myc, AR, E-cadherin and VEGF. Recent techniques for high-volume assessment of gene expression will accelerate the discovery of new predictive and prognostic molecules. The test of these and other markers of outcome will not only be their predictive potential but their ability to change the natural history of prostate cancer through directed intervention.

Conflict of interest statement

None declared.

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