The emergence of DNA methylation as a key modulator of aberrant cell death in prostate cancer

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Abstract

It is now well established that cancer cells exhibit a number of genetic defects in the machinery that governs programmed cell death and that sabotage of apoptosis is one of the principal factors aiding in the evolution of the carcinogenic phenotype. A number of studies have implicated aberrant DNA methylation as a key survival mechanism in cancer, whereby promoter hypermethylation silences genes essential for many processes including apoptosis. To date, studies on the methylation profile of apoptotic genes have largely focused on cancers of the breast, colon and stomach, with only limited data available on prostate cancer. Here we discuss the major developments in the field of DNA methylation and its role in the regulation of aberrant apoptosis in prostate cancer. The most significant advances have involved the discovery of apoptotic gene targets of methylation, including XAF1, (fragile histidine triad (FHIT), cellular retinol binding protein 1 (CRBP1), decoy receptor 1 (DCR1), decoy receptor 2 (DCR2), target of methylation-induced silencing 1 (TMS1), TNF receptor superfamily, member 6 (FAS), Reprimo (RPRM) and GLI pathogenesis-related 1 (GLIPR1). These genes are reported to be hypermethylated in prostate cancer and some offer potential as diagnostic and prognostic markers. We also introduce the concept of an ‘apoptotic methylation signature’ for prostate cancer and evaluate its potential in a diagnostic, prognostic and therapeutic setting.

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Introduction

Prostate cancer represents a global public health problem. Worldwide, it is the second most common noncutaneous cancer in men, accounting for ~10% of male cancers (Parkin et al. 2005). Prostate cancer is the second leading cause of cancer-related deaths among men in North America and Western/Northern Europe (Hughes et al. 2005). In recent years, 5-year survival rates for prostate cancer have been ranked third highest of all cancers (Toms 2004). Much of this improvement is due to an increase in the number of men being diagnosed with early-stage prostate cancer as a result of widespread use of prostate-specific antigen (PSA) testing. However, early diagnosis fails to predict accurately the outcome of individual patients. A major challenge is in distinguishing between clinically indolent prostate cancers and aggressive prostate cancers with the potential to kill the patient.

The molecular pathology of prostate cancer is complex; epidemiological studies have demonstrated familial clustering of prostate cancer suggesting that hereditary factors are important in the development of the disease in ~10% of cases (Porkka 2004). Emerging evidence strongly supports a role for environmental factors such as diet and inflammation in modifying the risk of prostate cancer (Nelson et al. 2002, De Marzo et al. 2007). Numerous molecular aberrations have been described, including chromosomal loss (8p, 10q, 13q, 16q) or gain (7p, 7q, 8q, Xq), gene amplification (MYC), mutations leading to increase or decrease in gene expression and functional mutations (Foley et al. 2004). Reduced expression of genes with important roles in cell cycle regulation and apoptosis (tumour protein 53; TP53), cell proliferation (phosphatase and tensin homolog; PTEN) and intracellular detoxification (glutathione S-transferase pi; GSTP1) have been implicated and are common throughout all stages of prostate cancer (Meyers et al. 1998).

Epigenetic modifications, specifically DNA hypermethylation, are believed to play an important role in
the down-regulation of genes important for protection against prostate cancer. In addition to classical genetic abnormalities, epigenetic modifications have emerged as a central driving force in the molecular pathology of prostate cancer (Perry et al. 2006).

It is now well established that cancer cells exhibit a number of genetic defects in apoptotic pathways, which aid the tumour during its evolution towards a carcinogenic phenotype. Analysis of the molecular mechanisms underlying defects in apoptotic pathways is an active area of research in prostate cancer. A number of studies have acknowledged a link between promoter hypermethylation and aberrant expression of certain apoptotic genes. In this review, we focus on DNA methylation and its role in the deregulation of apoptosis in prostate cancer. Both the fields of methylation and apoptosis will be discussed, establishing a link between these two important processes and their role in cancer progression. In the second part of this review article, we introduce and discuss the principle of an ‘apoptotic methylation signature’ for prostate cancer and its implication in diagnostic, prognostic and therapeutic applications.

The ‘Prima Donna’ of epigenetics: DNA methylation

Epigenetics can be defined as the mechanisms that initiate and maintain heritable patterns of gene expression without altering the sequence of the genome. There are several layers of epigenetic complexity including histone modifications, chromatin remodelling, micro-RNAs and DNA methylation. The latter being the most thoroughly studied to date (Esteller 2006).

DNA methylation has been described as the ‘Prima Donna’ of epigenetics, highlighting its central importance in epigenetic inheritance (Scarano et al. 2005). It refers to the covalent addition of a methyl group to the carbon at position 5 of the cytosine ring, by a family of DNA methyltransferase (DNMT) enzymes, resulting in 5-methylcytosine (Razin & Riggs 1980). In mammalian DNA, 5-methylcytosine is found in ~4% of genomic DNA, primarily at cytosine–guanine dinucleotides (CpGs; Baylin 2005). These CpG sites are non-randomly dispersed throughout the genome, concentrated in hot spots or CpG-rich regions known as CpG islands. Approximately half of all human genes are estimated to contain a 5' or promoter CpG island (Larsen et al. 1992, Wang & Leung 2004). CpG methylation is a key regulatory mechanism of the genome and plays a central role in diverse biological processes. Mice knockouts of the DNMT genes are lethal, suggesting a vital role for DNA methylation in normal mammalian development (Li et al. 1992, Okino et al. 2007). DNA methylation plays a significant role in suppressing parasitic repetitive elements, such as Alus and LINEs (both rich in CpGs), which have been incorporated into many transcriptional units of active genes (Yoder et al. 1997). As a general rule, CpG islands are usually unmethylated in normal tissue; with the exception of CpG islands at the promoters of genes on the inactive X chromosome, genes subject to parental imprinting, germline-specific genes and certain tissue-specific genes (Esteller 2005).

DNA methylation inhibits transcriptional initiation by i) directly blocking transcription factor binding to the promoter: NF-κB for example, is a transcription factor whose binding has been shown to be inhibited by DNA methylation (Bednarek 1993) and ii) indirectly by promoting histone deacetylation resulting in chromatin condensation (Bird 2002). The latter is more complex and deserves some discussion. Active genes usually lack promoter methylation and are generally associated with histone lysine (K), acetylation and histone methylation at H3K4, H3K36 and H3K79. Inactive genes, in contrast, are often hypermethylated at their promoters, while histone deacetylation favours interactions between adjacent nucleosomes, which may lead to chromatin condensation and transcriptional repression (Thiagalingam et al. 2003). Histone methylation at distinct residues can also have repressive effects on gene expression such as H3K9, H3K27 and H4K20 (Klose & Zhang 2007). There is evidence that H3K9 methylation can serve as a DNA methylation signal (Fuks 2005). A recent study by Smallwood et al. reports that heterochromatin protein 1 (HP1) mediates communication between histone modifications and DNA methyltransferases. This study supports a model whereby HP1 is attracted to euchromatic genes in regions where H3K9 has been methylated by the histone methyl transferase G9a. The HP1 molecule appears to act as an adaptor, which binds to DNMT1 and enhances its DNA methyltransferase activity, resulting in increased cytosine methylation of the surrounding DNA (Brenner & Fuks 2007). Studies have also shown that G9a activity can be enhanced by DNMT1 (Esteve et al. 2006). Smallwood et al. (2007) argue that the evidence suggests a mechanism whereby DNMT1, HP1 and G9a form a positive feedback loop, which helps control the silencing of genes.

Gene methylation in cancer

During oncogenic transformation, simultaneous gain (hyper) and loss (hypo) of methylation occurs. Global
Apoptosis

Programmed cell death or apoptosis is an evolutionary conserved and genetically regulated biological process that plays an important role in the development and homoeostasis of multicellular organisms (Creagh et al. 2003). Apoptosis is classically defined by a characteristic set of morphological changes that occur in cells, including blebbing of the plasma membrane, cell shrinkage, condensation and fragmentation of the nucleus, formation of apoptotic bodies and DNA fragmentation (Kerr 1972).

A major component of the mammalian cell death machinery is the caspase (cysteine aspartate-specific proteases) family of proteases. Currently 14 mammalian members of this family (caspase-1–caspase-14) have been identified. All known caspases contain an active site cysteine (cys) residue and cleave after aspartate acid (asp) residues. Caspases are typically synthesized in normal cells as enzymatically inert zymogens (enzymes containing an N-terminal prodomain, a P20 and P10 domain; Hengartner & Bryant 2000), which require autoproteolytic cleavage or cleavage by other caspases at specific asp residues to achieve their active configuration (Vermeulen et al. 2005). Caspases important for apoptosis can be further divided into two subgroups (initiators and effectors) on the basis of the lengths of their prodomain and relative position in the apoptotic caspase cascade (Creagh & Martin 2001). Upon activation, caspases with long prodomains (caspase-2, -8, -9 and -10) function upstream in the signal transduction pathway, as initiators, proteolytically activating downstream effector caspases 3, 6 and 7, which contain a short prodomain (Vermeulen et al. 2005). These downstream executioner caspases have been shown to be chiefly responsible for the majority of the intracellular caspase-induced cleavage that results in cell death (Earnshaw et al. 1999).

Death receptor pathway: caspase activation

Two main pathways of cell death have now been elucidated: a death-receptor-mediated pathway and a mitochondrial-mediated pathway. The death receptors are a subset of the tumour necrosis factor (TNF) receptor family of cell surface molecules that possess a common motif within their cytoplasmic tails, called death domains. Receptor death domains are thought to be intimately involved in the recruitment of adaptor molecules, which, in turn, enlist caspases to the receptor complex. Upon ligand binding, death receptors such as Fas aggregate and form membrane-bound signalling complexes (Fig. 1). These complexes recruit through adaptor proteins (e.g. Fas-associated via death domain; FADD), several molecules of procaspase-8. The induced proximity together with a weak protease activity of procaspase-8 is sufficient to allow mutual cleavage and transactivation (Hengartner & Bryant 2000). It is believed that caspase-8 propagates the death signal by direct processing of other caspases (Slee et al. 1999). Caspase-3 is a putative substrate for caspase-8. Once activated, caspase-3 can further propagate the cascade by activation of caspase-6 and caspase-7.

Mitochondrial apoptotic pathway: caspase activation

The mitochondrion is not only the cell’s powerhouse but also a key player in programmed cell death. The signal hypomethylation activates proviral and transposon sequences leading to the disruption of their surrounding genes (Yoder et al. 1997, Chalitchagorn et al. 2004) and also leads to elevated mutation rates by destabilizing the genome and promoting loss of heterozygosity in regions containing tumour suppressors (Ehrlich 2002). A number of recent studies have shown that de novo promoter hypomethylation may result in the activation of proto-oncogenes (Guo et al. 2000, Tamada et al. 2001, Pakneshan et al. 2003, Ogishima et al. 2005).

The major focus of epigenetic research in human cancer over the past decade has been promoter hypermethylation. In fact, it is now thought that hypermethylation-driven gene inactivation is at least as common as, if not more frequent than, traditional mutational events in the development of certain cancers (Fraga & Esteller 2005). The large number of genes found to undergo hypermethylation in a wide variety of cancer types suggest a key role for DNA methylation changes in the initiation and progression of cancer (Esteller 2006).

Promoter hypermethylation and silencing of the associated genes is widespread in prostate cancer. Many of the genes that are targeted by the cells methylation machinery play a role in regulating apoptosis and important cellular functions such as cell growth, differentiation and DNA repair, while others are implicated in tumour metastasis and angiogenesis (Perry et al. 2006). The most significant gene that has been described is GSTP1, which has been found to be methylated in >90% of prostate cancers (Lin et al. 2001, Maruyama et al. 2002). In our own lab, we have focused on the insulin-like growth factor (IGF) pathway and have recently identified IGFBP3 as a novel target of methylation in prostate cancer (Perry et al. 2007b).
for the mitochondrial-mediated pathway can arise from a number of signals including DNA damage caused by ionizing radiation and from cytotoxic agents (Vousden & Lu 2002, Kim et al. 2004b).

The bcl-2 family of proteins plays a key role in mitochondrial-mediated apoptosis. The family consists of both pro- and anti-apoptotic proteins, the members of which can be classified based on four conserved regions known as bcl-2 homology (BH) domains into three main subclasses (Cory & Adams 2002). Anti-apoptotic proteins like bcl-2, bcl-xl, mcl-1, A1 and bcl-w are characterized by the presence of the four known BH domains. This class of anti-apoptotic proteins appears to exert control over mitochondrial permeability by stimulating ADP/ATP exchange, stabilizing the mitochondrial inner transmembrane potential and preventing the opening of a plethora of pro-apoptotic molecules including cytochrome c (Miller 1999).

The bcl-2 family also contains a number of pro-apoptotic members, which can be separated into two subclasses, the BH 1–3 domain containing members (Bax and Bak) and members that contain only one BH domain (The BH3 only class: Bim, Bik, Bad, Hrk, Noxa and Puma; Cory & Adams 2002). A number of the latter class of molecules are up-regulated by P53 induction in response to intracellular damage (Huang & Strasser 2000). With the exception of Bid, they are thought to, upon stimulation, translocate to the mitochondria and block the protective regulatory activity of anti-apoptotic bcl-2 members (Miller 1999). The presence of either Bax or Bak seems to be essential for apoptosis in many cell types. Bax and Bak are thought to trigger or contribute to permeabilization of the outer mitochondrial membrane, allowing efflux of apoptotic proteins such as cytochrome c out of the mitochondria into the cytosol. The mechanism of Bax/Bak-mediated cytochrome c release is controversial and a number of models have been proposed and reviewed elsewhere (Cory & Adams 2002). A dynamic equilibrium exists between pro-apoptotic and anti-apoptotic bcl-2 family members, whereby the relative concentrations of pro-/anti-apoptotic bcl-2 members may decide life or death of the cell (Cory & Adams 2002).
The release of cytochrome $c$ into the cytosol has dramatic consequences for the cell on two levels. First, cytochrome $c$ is an integral part of the mitochondrial electron transport chain that is required for ATP synthesis and secondly, cytochrome $c$ binds to and activates cytosolic Apaf-1 (apoptotic protease activating factor-1). Apaf-1 controls the activation of caspase-9. To enable caspase activation, Apaf-1 must first bind cytochrome $c$ and undergo dATP-dependent oligomerization prior to caspase-9 binding (Zou et al. 1999). With cytochrome $c$ and dATP bound, Apaf1 and caspase-9 assemble into a high molecular weight complex that has been termed the ‘apoptosome’ (Green & Kroemer 1998). The apoptosome facilitates the cleavage and hence activation of caspase-3, -6 and -7.

Crosstalk occurs between the two pathways at the level of caspases. For example, caspase-8 can catalyse the cleavage of Bid into two fragments, the C-terminal portion, which can then translocate to the mitochondria where it integrates into the outer membrane and promotes cell death by activating Bax and Bak, and it may also play a role in the inactivation of its pro-survival relatives (bcl-2 and bcl-xl; Cory & Adams 2002).

Caspase activation appears to be the preferred executor of cell death; however, in its absence or failure can be compensated for by many other default pathways. Some of the mitochondrial proteins released as a result of mitochondrial outer membrane potential (MOMP; AIF, HtrA2/Omi, endonuclease G) can promote caspase-independent death via a number of mechanisms (Kroemer & Martin 2005).

**The deregulation of apoptosis in prostate cancer**

Disruption of normal apoptotic pathways contributes to carcinogenesis via a number of mechanisms including loss of function of tumour suppressor genes (e.g. TP53, APAF1, FAS), epigenetic inactivation of members of different apoptotic signalling cascades (e.g. CASP8, APAF1, BAX), defects in execution pathways (e.g. caspase-3, caspase-6) and up-regulation of anti-apoptotic proteins (e.g. bcl-2, bcl-xl; Hanahan & Weinberg 2000).

In prostate cancer, a number of molecular changes have been identified that target different components of the apoptotic cascade. For instance, in vivo studies have demonstrated that bcl-2-overexpressing prostate carcinoma cell lines are associated with a reduced apoptotic response following treatment with various chemotherapeutic agents and ionizing radiation (Kyprianou et al. 1997). In patients with locally advanced or metastatic prostate cancer receiving hormonal therapy, bcl-2 over-expression is an adverse prognostic indicator (Colombel et al. 1993, Shabaik et al. 1995).

P53 induces cell cycle arrest or apoptosis in response to DNA damage, hypoxia, heat shock and many other cellular stresses (Wang et al. 2004). P53 controls the expression of several pro-apoptotic genes such as BAX, the cyclin-dependent kinase inhibitor CDKN1A and transformed 3T3 cell double minute 2 (MDM2). TP53 mutations are uncommon in early invasive prostate carcinoma but occur frequently in advanced stages of disease (Navone et al. 1993). A number of studies have indicated that co-staining for p53 and bcl-2 is a potential biomarker for prostate cancer progression, poor prognosis and recurrence (Moul et al. 1996, Matsushima et al. 1997).

Aberrant expression of a number of promiscuous members of apoptotic pathways (both intrinsic and extrinsic) have been implicated in prostate cancer. Tissue microarrays of normal and cancerous prostates have identified BCL-2, FLIP, clustrin (CLU), mothers against DPP homology 4 (SMAD4), death-associated protein (DAP) and p53-induced protein PIGPC1 (PERP) as down-regulated in prostate cancer (Ernst et al. 2002, Yu et al. 2004). Such data highlights the importance of both pro- and anti-apoptotic molecules in prostate carcinogenesis.

Human prostate cancers often present as heterogeneous tumours, with a mixture of androgen-dependent and androgen-independent tumour cells. Androgen ablation therapy is the primary treatment for metastatic prostate cancer. However, this treatment alone does not eliminate the androgen-independent cells. Several lines of evidence suggest that progression to androgen-independent disease is associated with resistance to apoptosis and altered expression of caspases and expression of inhibitor of apoptosis proteins (IAPs; McEleny et al. 2002). Understanding which apoptotic pathways become altered and by what mechanism, for example genetic or epigenetic, may provide a rationale for targeting the cell death machinery in hormone refractory prostate cancer.

Manipulation of apoptotic pathways is an attractive therapeutic approach for the treatment of prostate cancer. Investigation into the differential expression of key apoptotic genes, such as BCL-2 and TP53, in normal prostate versus prostate cancer has provided valuable insights into the apoptotic profile of prostate cancer cells. Such a profile may act as an important feature of tumour pathology that could influence diagnosis, prognosis, therapeutic response and disease progression. The challenge now is to further dissect the molecular mechanisms underlying the atypical expression of key apoptotic genes.
DNA methylation of apoptotic genes: a key survival mechanism in prostate cancer

Evasion of cell death is a key feature of cancer cell survival, whether the cell is proliferating, metastasizing or developing resistance to chemotherapeutic drugs (Jones 2001). Studies suggest that DNA methylation in human cancer cells can help inactivate apoptotic pathways at several points. Furthermore, there is increasing evidence to suggest that the methylation status of apoptotic genes can aid in the detection and prognosis of cancer. To date, the majority of methylation studies on apoptotic genes have focused on cancers of the bladder, stomach, colon and breast. In prostate cancer, limited data exist on the methylation status of apoptotic genes (Table 1).

XIAP-associated factor 1 (Xaf-1)

XIAP is a potent member of the IAP family and inhibits caspase cleavage and initiation of apoptosis. Xaf-1 was identified as a negative regulator of XIAP (Liston et al. 2001). Studies suggest that loss of XAF1 expression might be important for cancer cell survival via its deregulation of apoptotic pathways, whereby it provides a survival advantage through the relative increase of XIAP anti-apoptotic function (Lee et al. 2006). Promoter hypermethylation has been widely reported in colon cancer and gastric and renal cell carcinomas (Byun et al. 2003, Jang et al. 2005, Kempkensteffen et al. 2007). Methylation of specific promoter CpG sites in the XAF1 gene is tightly associated with down-regulation of XAF1 expression in urogenital cancer cell lines and primary tumours. In the prostate, XAF1 methylation has been reported in 35% (7/20) of primary tumours, and no (0/10) benign prostatic hyperplasia (BPH) lesions (Lee et al. 2006).

Fang et al. recently observed the absence of XAF1 mRNA in PC3 prostate cancer cells and expression of a truncated XAF1 transcript in LNCaP and DU145 prostate cancer cell lines. Interestingly, treatment with the DNA methylation inhibitor, 5-aza-2'-deoxycytidine, led to re-expression of the full length XAF1 transcript. These findings could indicate a possible role for DNA methylation patterns on alternative mRNA splicing, which may ultimately confer survival advantages for cancer cells.

Cellular retinol binding proteins 1 (CRBPs)

CRBPs are key mediators of retinoid activity, in which they act as specific transporters of the alcoholic form of vitamin A (retinol) within the cell. CRBP2 and CRBP3 are tissue specific, while CRBP1 is widely expressed (Folli et al. 2001). Studies have demonstrated that DNA hypermethylation of CRBP1 is a common event in human cancer (Esteller et al. 2002). Two studies have implicated CRBP1 hypermethylation in prostate cancer. In 2004, Jeronimo et al. first reported CRBP1 promoter hypermethylation in 42.2% (17/32) of prostate adenocarcinomas, 8.6% (3/35) of pre-invasive high-grade prostatic intraepithelial Neoplasm (HGPIN), in only 1/36 morphologically normal prostate tissue samples and in 0% (0/32) of BPH lesions. More recently, Suzuki et al. (2006) detected CRBP1 methylation at a similar frequency, in 34% (34/101) of prostate cancers. Furthermore, this group reported a significant correlation between CRBP1 hypermethylation and both stage and Gleason score in prostate cancer. However, neither of these studies established a correlation between CRBP1 promoter methylation and loss of expression, which suggests that an additional mechanism to DNA hypermethylation may be required for CRBP1 down-regulation (Jeronimo et al. 2004). It would be interesting to investigate whether the chromatin environment surrounding CRBP1 is in an inactive state through evaluating patterns of modifications. CRBP1 is postulated to promote apoptosis via its up-regulation of all trans-retinoic acid (ATRA) synthesis. Therefore, it is thought that loss of CRBP1 could disrupt a retinoic acid mediated apoptosis pathway and hence support prostatic tumour progression (Kuppumbatti et al. 2000).

Fragile histidine triad (FHIT)

Structural and biochemical studies indicate that tumour suppressor gene FHIT is a pro-apoptotic factor. FHIT may induce apoptosis by up-regulating Bak (Sard et al. 1999). Hypermethylation in the promoter region of FHIT has been reported in a wide variety of cancers (Kim et al. 2004a, Hong et al. 2005, Goldberg et al. 2006, Leal et al. 2007). FHIT methylation in prostate cancer has only been described in one report, at a low frequency of 15% (Maruyama et al. 2002).

DCR1 and DCR2

The decoy receptor DcR1 lacks an intracellular death domain while DcR2 contains a shortened non-functional death domain. The extracellular domains of both decoy receptors are known to compete with those of the death receptors DR4 and DR5 for TNF-related apoptosis-inducing ligand (Trail) binding (Fig. 1). As a result of this competition for Trail binding, DcR1 and DcR2 are believed to inhibit apoptosis induction by binding to Trail but failing to
<table>
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<th>Gene symbol</th>
<th>Role in apoptosis</th>
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<td><strong>Intrinsic pathway</strong></td>
<td></td>
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<tr>
<td>XAF1</td>
<td>Interferes with the caspase-inhibiting activity of XIAP</td>
<td>Methylated in prostate cancer cell lines LNCaP (left supraclavicular lymph node metastasis), PC3 (bone metastasis) and DU145 (brain metastasis) 35% primary prostate cancers 0% BPH</td>
<td>Fang et al. (2006) and Lee et al. (2006)</td>
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<td>CRBP1</td>
<td>Promotes apoptosis via up-regulation of ATRA synthesis</td>
<td>34–42.2% prostate adenocarcinomas 2.8% normal prostate 0% BPH</td>
<td>Jeronimo et al. (2004) and Suzuki et al. (2006)</td>
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<td>FHIT</td>
<td>FHIT-induced apoptosis may be a result of BAK up-regulation</td>
<td>15% prostate cancer 0% non-malignant prostate tissue (BPH prostatitis and histologically non-malignant tissue adjacent to tumour)</td>
<td>Maruyama et al. (2002)</td>
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<td>DCR1, DCR2</td>
<td>Decoy receptors that fail to induce apoptosis upon TRAIL binding</td>
<td>50% prostate cancer 0% histologically non-malignant tissue adjacent to tumour</td>
<td>Shivapurkar et al. (2004) and Suzuki et al. (2006)</td>
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<td><strong>Extrinsic pathway</strong></td>
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<td>TMS1</td>
<td>Induces apoptosis in caspase-dependent manner</td>
<td>Methylated in prostate cancer cell lines LNCaP, DU145, PC3, MDAPCa2b (Bone metastasis) and LAPC4 (derived from Human prostate xenograft). 47–65% primary prostate cancer 64% HGPIN 3–28% non-malignant tissue adjacent to tumour</td>
<td>Collard et al. (2006) and Das et al. (2006)</td>
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<td>FAS</td>
<td>Death receptor for the Fas ligand and induces apoptosis via indirect cleavage of caspase-8</td>
<td>Methylated in metastatic prostate cancer cell line DU145 12.5% prostate cancer</td>
<td>Santourlidis et al. (2001)</td>
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<td>P53 signalling pathway</td>
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<td>RPRM</td>
<td>Inhibits Cdc2-cyclin b1 activity</td>
<td>54% prostate cancer 28% non-malignant prostate tissue (BPH, histologically normal adjacent tissue)</td>
<td>Suzuki et al. (2006)</td>
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<td>GLIPR1</td>
<td>p53 target gene</td>
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<td>Ren et al. (2004)</td>
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XIAP, X-linked mammalian inhibitor of apoptosis protein; BPH, benign prostatic hyperplasia; ATRA, all-trans-retinoic acid; BAK, BCL-2-antagonist/killer; TRAIL, TNF-related apoptosis-inducing ligand; HGPIN, high-grade prostatic intraepithelial neoplasia.
execute apoptosis via their non-functional or missing death domains (Ashkenazi & Dixit 1999). Aberrant methylation of DCR1 and DCR2 genes has been reported in a number of tumour types (Shivapurkar et al. 2004). DCR1 and DCR2 were both methylated in 50% (25/50 and 23/46 respectively) of prostate tumours and 60% of prostate cancer samples had either DCR1 or DCR2 methylated. Interestingly, methylation of either DCR1 or DCR2 was correlated with improved prognosis (Shivapurkar et al. 2004) and a subsequent study showed that an unmethylated status of both genes predicted a poorer disease-free survival rate (Suzuki et al. 2006). DCR1 and DCR2 expressions are believed to be advantageous to tumour cells because they may aid the cells in evading cell death. It is currently unknown why silencing of decoy receptors would favour tumour progression and a number of theories have been postulated (Reviewed in Shivapurkar et al. 2004).

Target of methylation-induced silencing (TMS1)/apoptosis speck-like protein containing a CARD (ASC)

TMS1 also known as ASC is a pro-apoptotic tumour suppressor gene. The N-terminal pyrin domain of TMS1 appears to act as an activating adaptor for caspase-8 (Masumoto et al. 2003) and hence promotes caspase-dependent apoptosis. TMS1 is widely methylated in human cancers (Martinez et al. 2007, Zhang et al. 2007). Two recent reports have implicated methylation as a mechanism of TMS1 down-regulation in prostate cancer cell lines (Collard et al. 2006, Das et al. 2006). Biallelic hypermethylation and silencing of TMS1 has been shown in LNCaP cell line. Pharmacological demethylation with 5-aza-2’-deoxycytidine (5-aza-CdR) reactivated TMS1 expression. However, treatment with HDAC inhibitor failed to induce gene expression, suggesting that histone deacetylation does not play a role in down-regulation of TMS1 in prostate cancer cell lines (Das et al. 2006). Further investigation is required to properly evaluate the role of histone modification in TMS1 silencing.

TMS1 promoter hypermethylation has been implicated as an early event in prostate cancer development, detected at significantly higher frequencies in primary tumours (47–65%) and pre-invasive HGPIN (64% (7/11)) than non-malignant prostate (3–28%; Collard et al. 2006, Das et al. 2006, Suzuki et al. 2006). Of these three studies, only Suzuki et al. found a significant correlation between TMS1 hypermethylation and Gleason score ≥ 7. Interestingly, a study which investigated differences in methylation frequencies between different racial groups reported no difference in the methylation frequency of TMS1 between African American and Caucasian American tumour samples, but found that in African Americans; TMS1 was methylated at similar frequencies in prostate cancer and BPH (Das et al. 2006). Further studies are required to evaluate whether aberrant methylation patterns of TMS1 may contribute to differences between different racial groups in their susceptibility to prostate cancer.

FAS

As described previously, Fas protein induces apoptotic cell death upon Fas ligand binding. Santourlidis et al. (2001), analysed the methylation status of FAS promoter region in two cell lines (DU145 and LNCaP) and 32 prostatic carcinomas by restriction enzyme PCR analysis. The FAS gene was reported to be weakly hypermethylated in DU145 cells and in 12.5% of the prostate tumours (4/32). Following treatment with the DNA methyltransferase inhibitor 5-aza-CdR methylation of the FAS promoter decreased; however, induction of FAS mRNA was not detected. Three methylated CpG sites within the FAS promoter region are contained within a predicted binding site for NF-kB. Further study is warranted to establish whether FAS silencing is a result of inhibiting transcription factor binding rather than alterations in histone modifications. Investigation into the methylation of FAS in normal prostate samples is required to fully evaluate the potential role of FAS methylation as a prostate cancer biomarker.

Reprimo (RPRM)

RPRM is a putative mediator of p53-mediated cell cycle arrest at the G2 phase of the cell cycle (Ohiki et al. 2000). Aberrant methylation of RPRM has been reported in a number of cancer types (Hamilton et al. 2006, Sato et al. 2006). Suzuki et al. (2006) reported that 54% (55/101) of prostate cancers showed aberrant methylation of RPRM when compared with 28% (9/32) of non-malignant prostatic tissues. The authors argue that RPRM methylation status in addition to the methylation pattern in several other apoptotic genes could represent a potential biomarker of risk prediction in prostate cancer, the clinical relevance of which requires further investigation.

GLIPR1

GLI pathogenesis-related 1 (GLIPR1) was recently identified as a novel P53-regulated gene. GLIPR1 has
been shown to be down-regulated in prostate cancer compared with normal prostatic tissue (Ren et al. 2004). DNA methylation appeared to be a prevalent method of down-regulation and GLIPR1 gene expression could be restored by treatment with a demethylating agent. DNA from 13 tumour tissue samples had a higher frequency of methylation in all of the CpG dinucleotides in the promoter CpG when compared with histologically normal tissue adjacent to each tumour. Analysis of the mean frequency of three CpG dinucleotides close to the ATG start site showed significantly higher methylation frequencies in tumour tissue when compared with normal prostate in 13 paired normal and tumour specimens. Unfortunately, there are only limited data available on the biological role of GLIPR1, but this report suggests it may play an important role in prostate cancer progression. To date, this is the only report of methylation of GLIPR1 in cancer (Ren et al. 2004).

In summary, frequent hypermethylation has been demonstrated for most of these apoptotic-related genes in a wide variety of tumour types. However, to date, only a small number of studies have been carried out in prostate cancer. Further validation of the methylation profile of these targets is necessary in order to investigate their potential as prostate cancer biomarkers and to understand their role in prostate carcinogenesis. Investigation into the methylation of these genes in larger numbers of prostate cancer tissue samples using more sensitive methods such as quantitative Methylation-Specific PCR may prove more informative, allowing for the relative level of methylation in the particular sample to be quantified. In addition, new developments in methylation array technologies will allow for hundreds of CpG islands to be rapidly screened in large archives of patient material.

In the case of FHIT, RPRM and DCR1/2, there are no reports of gene expression analysis in prostate cancer cell lines or tissue. To determine if DNA methylation is associated with gene silencing, it would be important to examine the mRNA levels of these genes. In addition, there is an intimate relationship between DNA methylation and histone modifications (particularly acetylation and methylation) in gene silencing, suggesting that all avenues of epigenetic marks should be investigated in order to understand their dynamic control on gene expression in prostate cancer aetiology. Understanding the epigenetic mechanisms involved in gene silencing may shed insight into cancer progression as highlighted by a recent report by Okino et al. (2007). This group investigated chromatin changes associated with GSTP1 silencing. GSTP1 is hypermethylated and silenced in >90% of prostate cancers and is one of the most widely studied targets of DNA methylation in prostate cancer. The authors employed the LNCaP cell line (with biallelic hypermethylation and silencing of GSTP1) as a model of prostate cancer and found that the GSTP1 promoter was not associated with activating histone modifications (acetylation at H3 and H4 and di- or tri- methylation at H3K4), whereas in non-cancerous cell lines RWPE1 and PWR1E, the promoter of actively expressed GSTP1 was unmethylated and associated with high levels of the activating marks. There is still some debate as to which epigenetic modification becomes altered first during prostate carcinogenesis (Stirzaker et al. 2004, Okino et al. 2007); nevertheless, thorough investigation into both DNA methylation and histone modification may help us to understand the process of epigenetic silencing.

Identifying an apoptotic methylation phenotype

To date, only one study has screened a panel of apoptotic genes in prostate cancer with a view towards generating a ‘methylator phenotype’. This study by Suzuki et al. (2006) examined the methylation status of 12 genes, all of which are directly or indirectly involved in apoptosis. The methylation status of the 12 genes was examined in 101 prostate cancer tissues and 33 non-malignant prostate tissues. Methylation frequencies of the genes were RPRM 54%, TMS1 47%, DCR1 45%, RRAD 37%, DCR2 37%, CRBP1 34%, HPP1 32%, RIZ1 31%, DRM/GREM1 21%, SOC1 20%, DR4 5% and DR5 1%. Analysis of clinicopathologic data revealed that methylation of RPRM and TMS1 correlated with preoperative serum PSA levels, while methylation of TMS1 and CRBP1 was significantly associated with Gleason score ≥7. Multivariate analysis of age, Gleason score, PSA, stage, and DCR1, DCR2 and TMS1 methylation was preformed. Results indicated that stage, unmethylation of both decoy receptors DCR1 and DCR2 and hypermethylation of TMS1 were shown to be independent prognostic factors for poorer disease-free survival. This study also scrutinized the panel of apoptotic-related genes for simultaneous multiple gene hypermethylation in prostate cancer, as has been reported previously in other cancer types (Toyota et al. 1999, Ueki et al. 2000). The authors found no evidence of a CpG island methylator phenotype (CIMP) or simultaneous multiple-gene hypermethylation (Suzuki et al. 2006).

Assessment of the expression levels of the apoptotic genes shown to be methylated is required to establish a silencing effect of promoter hypermethylation in prostate cancer. Furthermore, the paper by
Suzuki et al. (2006) was conducted on a mixed ethnic group. It has been shown that differences in promoter hypermethylation exist for certain genes between different racial groups (Woodson et al. 2003, 2004). Differences with respect to apoptosis have also been reported whereby apoptosis in malignant prostate cells were significantly higher in African Americans than Caucasian men (Guo et al. 2000). Furthermore, down-regulation of anti-apoptotic Bcl-2 expression was also observed in prostate tumours from African-American men compared to Caucasians (Guo et al. 2000). As the incidence of prostate cancer varies tremendously between different ethnic groups, it is plausible that mixed ethnic samples may affect methylation results. Comparing a prostate cancer methylation profile of Caucasians, Asians and Africans may help to elucidate some of the molecular differences underlying the more aggressive prostatic tumour phenotypes associated with patients of African ethnicity.

Clinical relevance of a ‘methylation signature’ of apoptosis

To date, a number of studies have highlighted the potential of methylation profiling in a diagnostic and prognostic setting. A large number of these studies have focused on GSTP1. The high prevalence of GSTP1 promoter hypermethylation in early prostate carcinogenesis has lead to the development of a GSTP1 hypermethylation assay for the detection of prostate cancer (Henrique & Jeronimo 2004). However, the effectiveness of GSTP1 alone as an early detection biomarker is limited and increasing evidence suggests that combining GSTP1 with other genes shown to be methylated in prostate cancer could be more powerful as a diagnostic and prognostic biomarker (Esteller et al. 2001, Bastian et al. 2005). An extensive study that quantified methylation of 16 different genes, described a four-gene panel of GSTP1, APC, PTGS2 and NDR1 that could accurately distinguish primary prostate cancer from benign prostate tissue with 92% specificity (Yegnasubramanian et al. 2004). Development of an apoptotic methylation signature, consisting of a panel of genes, whose aberrant expression is important for prostate cancer development and progression, may also prove beneficial as a tumour biomarker in prostate cancer. Recently we have undertaken a combined bioinformatics and wet-lab approach, to identify novel apoptotic gene targets for methylation in prostate cancer (Murphy et al. Abstract: DMMC International Workshop- Epigenetics:From Mechanisms to medicine; 25–26 June 2007, Dublin); an approach we have used previously to identify novel targets of hypermethylation such as IGFBP3 (Perry et al. 2007b). Such research is in its infancy, but with further identification of markers and standardization of techniques, it may be possible to significantly increase the sensitivity of detecting low volume disease (Perry et al. 2006). Furthermore, methylation patterns can be detected in DNA isolated from body fluids of cancer patients, which would provide a non-invasive means of diagnosis (Gifford et al. 2004).

A number of recent studies have highlighted the potential of methylation analysis of either individual genes or of a panel of genes as predictive or prognostic markers (Maruyama et al. 2002, Suzuki et al. 2006). Maruyama et al. showed that methylation of RASSF1A, GSTP1, RARβ2 and CDH13 genes correlates with clinicopathological features of poor prognosis. Suzuki et al. found that the methylation profile of a panel of apoptotic genes (TMS1, DCR1, DCR2 and CRBP1) correlates with clinicopathological features of prognosis. Therefore, analysis of an ‘apoptotic methylation signature’ in prostate cancer may help elucidate a panel of genes that help stratify patients into low- and high-risk groups.

Methylation and therapeutic targeting

Could methylation profiling of apoptotic genes in prostate cancer help elucidate novel therapeutic targets for drug-resistant disease? Recently, evidence suggests that hypermethylation of pro-apoptotic genes may result in conventional chemotherapeutic drug resistance. Methylation of the potent pro-apoptotic gene APAF1, for example, in malignant melanoma cells, has been shown to enhance cell survival during chemotherapy (Jones 2001). CASP8 represents another gene whose methylation may contribute to chemoresistance. Pharmacological demethylation of cancer cell lines exhibiting aberrant caspase-8 expression due to hypermethylation of the CASP8 gene, sensitized cells to both death receptor triggered apoptosis and drug-induced apoptosis (Fulda et al. 2001). Methylation of other genes implicated in the control of apoptosis may also have the potential to confer chemosensitivity. In this setting, determining the methylation status of a panel of apoptotic genes could be used to design novel therapeutic strategies for individual patients (Teodoridis et al. 2004b). Treatment with both methylation inhibitors and chemotherapeutic agents may prove beneficial in the treatment of resistant disease (Strathdee et al. 1999, Plumb et al. 2000). Currently, a number of studies have focused on the development of small molecular inhibitors of methylation and have produced a number of drugs, such as
5-azacytidine, 5-aza-2'-deoxycytidine, procaine, hydralazine and zebularine (Brueckner & Lyko 2004). These DNMT inhibitors, once incorporated into DNA, complex with and inactivate all three forms of DNA methyltransferase (Teodoridis et al. 2004b). However, as discussed, DNA methylation alone is often insufficient for gene silencing, with strong evidence supporting an integral role for histone deacetylation and chromatin remodelling in the silencing process (Bird & Wolffe 1999).

Histone deacetylase (HDAC) inhibitors have been used synergistically with demethylating agents to activate expression of methylated genes (Cameron et al. 1999). Studies suggest that this mechanism of drug administration is more potent than either DMNT inhibitors or HDAC inhibitors alone (Teodoridis et al. 2004a). For example, co-treatment of androgen receptor-negative cell line DU145 with 5-azacytidine (5-aza-CR) and histone deacetylase (HDAC) inhibitor Trichostatin A is most effective in restoring functional expression of the AR gene and its downstream targets, compared with either agent alone (Jarrard et al. 1998, Nakayama et al. 2000). Treatment with a demethylating agent in conjunction with HDAC inhibitors may be an effective anti-cancer approach without the need for conventional chemotherapeutic drugs in selected patients. It is important to note that both DNMT inhibitors and HDAC inhibitors have the potential to cause global genome hypomethylation, resulting in many possible side effects. However, DNMT inhibitors are thought to inhibit tumour growth by specifically reversing the repression of tumour suppressor and cell cycle genes that are aberrantly silenced by methylation (Issa 2003).

**Conclusion**

In conclusion, the ‘Prima Donna’ of epigenetics, DNA methylation, is an emerging field of research in prostate cancer. While investigation into the methylation status of genes as biomarkers for diagnostic, prognostic and therapeutic approaches are in its infancy, a number of genes have been identified, which show significant promise. The challenge is to rigorously evaluate potential markers using standardized techniques and to test their limit of detection and specificity for prostate cancer over other cancers and benign urological diseases. Deciphering the methylation profile of an integral cellular process such as apoptosis, whose dysregulation is key for tumour progression, could yield a biomarker signature. To date, only limited data are available on the methylation status of apoptotic genes in prostate cancer. Despite this, preliminary results are encouraging and further investigation is justified. With increases in the sensitivity of methods in detecting methylation, such as denaturing high performance liquid chromatography analysis (Perry et al. 2007a) and Quantitative Methylation-Specific PCR (QMSP), it is feasible that further apoptotic genes which are silenced by methylation are likely to emerge and their role in detection and prognosis in prostate cancer may be established. Studies on targets such as GSTP1 have recognized an important role for gene methylation pattern analysis in both a diagnostic and prognostic setting in prostate cancer. Methylation studies have also contributed to a new approach in cancer drug design, exploiting the reversible nature of DNA methylation, as a therapeutic target.

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