The emerging roles of DNA methylation in the clinical management of prostate cancer

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Abstract

Aberrant DNA methylation is one of the hallmarks of carcinogenesis and has been recognized in cancer cells for more than 20 years. The role of DNA methylation in malignant transformation of the prostate has been intensely studied, from its contribution to the early stages of tumour development to the advanced stages of androgen independence. The most significant advances have involved the discovery of numerous targets such as GSTP1, Ras-association domain family 1A (RASSF1A) and retinoic acid receptor β2 (RARβ2) that become inactivated through promoter hypermethylation during the course of disease initiation and progression. This has provided the basis for translational research into methylation biomarkers for early detection and prognosis of prostate cancer. Investigations into the causes of these methylation events have yielded little definitive data. Aberrant hypomethylation and how it impacts upon prostate cancer has been less well studied. Herein we discuss the major developments in the fields of prostate cancer and DNA methylation, and how this epigenetic modification can be harnessed to address some of the key issues impeding the successful clinical management of prostate cancer.

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Introduction: addressing problems with the clinical management of prostate cancer

Prostate cancer (CaP) has a unique set of problems associated with its early detection, diagnosis and treatment that might be aided by the complementary use of molecular markers such as DNA hypermethylation. CaP is now the most commonly diagnosed non-cutaneous malignancy and the second leading cause of cancer-related deaths among men in the USA and Europe (Jemal et al. 2005a). With ageing of the Western male population and the spread of Western lifestyle (a major risk factor), CaP is an escalating international health problem.

The diagnosis of early-stage CaP is imperative for its successful management, since the inevitable emergence of androgen insensitivity in late-stage tumours leads to significant mortality. However, the methods employed in diagnosis (serum prostate-specific antigen (PSA) measurement and digital rectal examination, confirmed by histological examination of biopsy specimens) are confounded by significant limitations. Although PSA is widely regarded as one of the best serum tumour markers, its poor specificity for CaP means that up to two-thirds of men with an elevated PSA (>4 ng/ml) have normal histology upon biopsy (Frankel et al. 2003). Subjecting all men with an elevated PSA to transrectal ultrasound (TRUS)-guided biopsy causes unnecessary anxiety in some men and risks detecting ‘clinically insignificant’ tumours that may otherwise never require treatment. A further consideration is that up to one-third of men with CaP have serum PSA levels below 4 ng/ml and would not be identified solely by such a screening programme (Hernandez & Thompson 2004). The remarkable heterogeneous and multifocal appearance of CaP between patients and even within an individual’s neoplasm makes collection of homogenous tumour tissue for diagnosis difficult. TRUS-guided needle biopsies may also miss microscopic foci of cancer, giving false negative results (Epstein & Potter 2001) and creating a further dilemma for those patients whose PSA levels continue to rise.

The treatment of CaP is difficult, in that only a small proportion of men diagnosed will have the
aggressive form of the disease. Prostate tumours can be very slow growing and as such many men die with and not because of their CaP (Sakr et al. 1994). However, we currently lack the necessary tools to discern between latent disease with little likelihood of clinical manifestation and aggressive tumours that are more likely to metastasise to potentially lethal disease.

There is a considerable amount of data that suggests that DNA hypermethylation may be useful for the early detection and diagnosis of CaP. In addition, a growing body of evidence shows an association between specific gene methylation and clinicopathologic indicators of poor prognosis in CaP. We present a broad review of the major findings of these studies. In addition, we discuss recent investigations of genome hypomethylation in CaP and the potential of the epigenome as a target for novel therapeutic strategies.

The marvellous world of DNA methylation

Epigenetic mechanisms such as DNA methylation and histone modifications play important roles in normal developmental processes, in gene imprinting and in human carcinogenesis. Together, they produce changes in chromatin structure that may affect gene expression in a heritable manner without directly altering the genetic code.

DNA methylation describes the covalent modification of DNA through the bonding of a methyl group to cytosine, in the palindromic dinucleotide doublet cytosine guanine (CpG; Bird 1986). While the majority of CpG dinucleotides in the human genome are dispersed across retrotransposons or throughout the coding regions and introns of genes, up to 15% occur in CpG islands (Bird 1986, Yoder et al. 1997). CpG islands are 200–2000 bp stretches of DNA with a GC content >50% (Gardiner-Garden & Frommer 1987, Takai & Jones 2002). They occur in the s′ region, including the promoter, untranslated region and first exon of approximately half of all human genes (Wang & Leung 2004). Between 70 and 90% of all CpGs are methylated in human DNA from normal somatic cells; methylation is catalysed by the DNA methyltransferases (DNMTs) and can be reversed by demethylases or by treatment with demethylating drugs such as 5-azacytidine (5-aza-C; Goffin & Eisenhauer 2002).

Promoter-CpG islands, however, are typically unmethylated and are conducive to gene expression. Methylation of promoter-CpG islands can result in transcriptional silencing of genes through a number of mechanisms, including (i) prevention of methylation-sensitive transcription factor binding (Iguchi-Ariga & Schaffner 1989, Hark et al. 2000) and (ii) attraction of methyl-cytosine proteins that interact with histone deacetylases (HDACs) and chromatin remodelling factors leading to chromatin condensation and an inaccessibility of the promoter to transcription factors (Ng & Bird 1999, Burgers et al. 2002). Therefore, in addition to classical genetic mechanisms involving chromosomal alterations and point mutations, genes can be functionally activated or inactivated by DNA methylation.

An imbalance of DNA methylation occurs in human neoplasia through both gain (hypermethylation) and loss (hypomethylation). These changes manifest as (i) de novo promoter hypermethylation, which typically occurs in a focal fashion, affecting genes with important cell regulatory functions, leading to their transcriptional inactivation, (ii) promoter hypomethylation, which may result in the activation of proto-oncogenes (Pakneshan et al. 2003), (iii) genome-wide hypomethylation, which can activate proviral and retrotransposon sequences and lead to disruption of their surrounding genes (Yoder et al. 1997) and (iv) loss of imprinting (Jarrard et al. 1995). In addition, the density and ‘spread’ of CpG dinucleotide methylation, the level of methylation within a tumour (methylated/unmethylated DNA copy number) and the frequency of methylation (percentage of tumours that are methylated at a given locus) are all important mediators of methylation effects in carcinogenesis.

It is important to note that changes in DNA methylation are often accompanied by changes in chromatin structure, modulated through histone modification. Thus, many malignant changes attributed to alterations in DNA methylation status probably also involve changes in histone biochemistry. In fact, methylation may not be the initial event in triggering epigenetic silencing, but may occur as a consequence of prior gene silencing, locking chromatin in a repressed state (Stancheva 2005).

Potential for DNA methylation in the clinical arena

There are several characteristics of DNA hypermethylation that make it advantageous as a cancer biomarker. Firstly, DNA methylation is a stable, positive signal, which can be tested in a variety of sample types without special handling procedures for the procurement of biological specimens. This attribute has only been strengthened by the recent
surge in development of techniques to analyse methylation. For example, quantitative real-time methylation-specific PCR (QMSP) is now widely employed to compare the proportion of methylated target between samples. The basis of this approach is bisulphite modification of genomic DNA, which converts unmethylated cytosines to uracils but methylated residues are protected from conversion. PCR oligonucleotides are designed to specifically amplify modified, methylated template. The relative levels of methylated promoter DNA in each sample are determined by the ratio of the target gene to an internal reference gene (that amplifies modified template independent of methylation status). QMSP can reliably detect methylation in the presence of a 10 000-fold excess of unmethylated alleles (Eads et al. 2000), which makes this technique particularly suited for clinical studies where sensitive detection is necessary owing to scarce tumour DNA or dilution by excess normal DNA. QMSP is now being employed to quantify methylation levels in laser-capture microdissected material, paraffin-embedded archival material and tumour DNA shed by cancer cells into bodily fluids such as blood and urine. These latter samples are easily obtainable from men with CaP, and present the opportunity to detect and monitor methylation changes via non-invasive approaches.

The high prevalence of DNA hypermethylation in human cancers, including prostate (Tables 1 and 2), is an advantage for identification of potential methylation diagnostic and prognostic biomarkers (Esteller et al. 2001, Yan et al. 2001). Unique profiles of methylated promoter-CpG islands identified in specific tumour types have also been reported (Costello et al. 2000, Esteller et al. 2001). This is an important attribute given that a major consideration for an early detection biomarker should be its inherent ability to distinguish CaP from other tumour types.

Tumour specificity is an obvious factor when considering promoter methylation as a cancer biomarker. However, methylation of a number of genes such as Ras-association domain family 1A (RASSF1A), cellular retinol-binding protein 1 (CRBP1), adenomatous polyposis coli (APC) and retinoic acid receptor β2 (RARβ2) has been reported at similar frequencies in CaP and in benign prostatic hyperplasia (BPH), an age-related non-cancerous enlargement of the prostate (Chu et al. 2002, Jeronimo et al. 2004b, 2004c). QMSP enables the quantification of methylated gene copies, which may allow the discrimination between methylated benign tissue and carcinoma. For example, in the study by Jeronimo et al. (2004c), APC was reported as methylated in 100% of tumours and 87% of BPH samples, but the median levels of methylation detected (expressed as the ratio of (methylated APC/β-actin) × 1000) were significantly different, at 86 and 0.7, respectively. Therefore, although methylation of certain genes occurs in benign prostate, by employing an empirical cut-off value it is possible to differentiate between high amounts of methylation in tumour DNA and low levels in benign conditions.

Considerable difficulty arises however, when attempting to compare independent studies, particularly with regards to interpreting highly divergent methylation frequencies reported in both tumour and benign specimens. Certainly, a degree of variation is due to biological discrepancies between sample populations, such as the purity of specimens, differences between tumours – in particular stage and grade of disease – and perhaps even geographical variation. In addition, the genomic position of CpG dinucleotides examined (or the location of primer- and probe-binding sites) may significantly affect results. Methylation patterns at promoter-CpG islands are typically not homogeneous. Instead, there are distinct peaks of methylation that may reflect the nucleosome positioning or chromatin structure of DNA and may be influenced by factors such as the location of transcription factor-binding sites, which are often differentially methylated between expressing and non-expressing promoters (Nakagawa et al. 2001, Song et al. 2002). However, a large proportion of disparity undoubtedly arises from a lack of technical consistency between reports. In particular, there is substantial variation in methylation frequencies depending on whether quantitative or conventional methylation-specific PCR is employed. QMSP is more stringent because of the additional CpG sites that can be examined in a single assay due to the fluorescently labelled hybridization probe, which has to anneal correctly between the two primers. Yet, low annealing temperatures and greater numbers of PCR cycles adapted by some studies have led to the misconception that methylation-specific PCR is a more sensitive technique than QMSP.

Hypermethylation biomarkers for early detection

In determining relevant early detection biomarkers, it is important to understand the molecular mechanisms implicated in CaP initiation. Therefore, investigations have focused on premalignant lesions as well as early-stage disease. High-grade prostatic
Table 1 Genes hypermethylated at a high frequency (>50%) in prostate cancer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stage</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Carcinogen metabolism</strong></td>
<td></td>
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<tr>
<td>GSTP1</td>
<td>HGPIN, primary and metastatic</td>
<td>Lin et al. (2001b), Maruyama et al. (2002), Yamanaka et al. (2003), Jeronimo et al. (2004c), Kang et al. (2004), Singal et al. (2004a), Woodson et al. (2004a), Yegnasubramanian et al. (2004)</td>
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<tr>
<td><strong>Negative regulator of tumour progression</strong></td>
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<tr>
<td>S100A2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HGPIN, primary and metastatic</td>
<td>Jeronimo et al. (2004c), Rehman et al. (2005)</td>
</tr>
<tr>
<td>APC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HGPIN, primary and metastatic</td>
<td>Maruyama et al. (2002), Jeronimo et al. (2004c), Kang et al. (2004), Yegnasubramanian et al. (2004)</td>
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<td><strong>Steroid hormone receptors</strong></td>
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<tr>
<td>ERα&lt;sup&gt;b&lt;/sup&gt;</td>
<td>All stages, increasing frequency with disease progression</td>
<td>Sasaki et al. (2002), Li et al. (2000)</td>
</tr>
<tr>
<td>ERβ</td>
<td>Primary</td>
<td>Sasaki et al. (2002)</td>
</tr>
<tr>
<td>ERβ&lt;i&gt;II&lt;/i&gt;</td>
<td>Primary, decreasing frequency in distant metastases</td>
<td>Nojima et al. (2001), Sasaki et al. (2002), Zhu et al. (2004)</td>
</tr>
<tr>
<td>RARβ&lt;sup&gt;2&lt;/sup&gt;</td>
<td>HGPIN, all stages, increasing frequency with disease progression</td>
<td>Nakayama et al. (2001), Maruyama et al. (2002), Yamanaka et al. (2003), Jeronimo et al. (2004b), Singal et al. (2004a), Woodson et al. (2004b)</td>
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<tr>
<td><strong>Scaffolding protein</strong></td>
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<td>Caveolin-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Primary</td>
<td>Cui et al. (2001), Woodson et al. (2004a)</td>
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<td><strong>Cell adhesion</strong></td>
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<td>CD44</td>
<td>All stages, increasing frequency with disease progression</td>
<td>Lou et al. (1999), Verkaik et al. (2000), Kito et al. (2001), Woodson et al. (2003), Singal et al. (2004a), Woodson et al. (2004a), Woodson et al. (2004b)</td>
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<tr>
<td>TIG1</td>
<td>All stages, increasing frequency with disease progression</td>
<td>Zhang et al. (2004)</td>
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<tr>
<td><strong>Cell growth/proliferation</strong></td>
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<tr>
<td>MDR1</td>
<td>All stages, increasing frequency with disease progression</td>
<td>Enokida et al. (2004), Yegnasubramanian et al. (2004)</td>
</tr>
<tr>
<td>RASSF1A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HGPIN, primary and metastatic</td>
<td>Kuzmin et al. (2002), Maruyama et al. (2002), Jeronimo et al. (2004c), Kang et al. (2004), Singal et al. (2004a), Woodson et al. (2004a), Woodson et al. (2004b), Yegnasubramanian et al. (2004)</td>
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<tr>
<td>ZNF185</td>
<td>All stages, increasing frequency with disease progression</td>
<td>Vanaja et al. (2003)</td>
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<tr>
<td>NTRK2</td>
<td>Primary</td>
<td>Yamada et al. (2004)</td>
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<td>HIN-1</td>
<td>Primary and metastatic</td>
<td>Krop et al. (2004), Shigematsu et al. (2005)</td>
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<tr>
<td>14-3-3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Primary</td>
<td>Lodygin et al. (2004), Mhawech et al. (2005)</td>
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<tr>
<td>hSPRY2</td>
<td>Primary</td>
<td>McKie et al. (2005)</td>
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<tr>
<td><strong>Normal development</strong></td>
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<tr>
<td>EDNRB&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Primary, metastatic</td>
<td>Nelson et al. (1997), Jeronimo et al. (2003), Singal et al. (2004a), Woodson et al. (2004a), Woodson et al. (2004b), Yegnasubramanian et al. (2004)</td>
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<tr>
<td><strong>Apoptosis</strong></td>
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<tr>
<td>RTVP1/GLIPR</td>
<td>Primary</td>
<td>Ren et al. (2004)</td>
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<tr>
<td>CRBP1</td>
<td>HGPIN, primary</td>
<td>Jeronimo et al. (2004a), Jeronimo et al. (2004c)</td>
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<tr>
<td>DcR1/TRAILR3</td>
<td>NA</td>
<td>Shivapurkar et al. (2004)</td>
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<tr>
<td><strong>Inflammation</strong></td>
<td></td>
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<tr>
<td>PTGS2/COX2</td>
<td>HGPIN, Primary and metastatic</td>
<td>Kang et al. (2004), Yegnasubramanian et al. (2004)</td>
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</table>

NA, not available. Primary prostate cancer denotes both organ-confined and advanced tumours that extend beyond the prostatic capsule.

<sup>a</sup>Different methylation frequencies have been reported for these genes, but may reflect analysis of different CpG sites and/or sensitivity disparities between technologies.

<sup>b</sup>Methylation of these genes occurs at similar frequencies in malignant and non-malignant tissue, although often at exonic CpG sites and at lower densities in non-tumour tissue.

<sup>c</sup>Methylation has only been assessed in samples with loss of gene expression.
Promoter hypermethylation is an early event in carcinogenesis in many human malignancies (Costello et al. 2000). The frequency of methylation of genes such as GSTP1, APC, RASSF1A, RARβ2, CRBP1, tissue inhibitor of metalloproteinase 3 (TIMP3), 0–6 methylguanine DNA methyltransferase (MGMT) and prostaglandin endoperoxidase

| Table 2 Genes hypermethylated at a low to moderate frequency (<50%) in prostate cancer |
|-----------------------------|-----------------------------|-----------------------------|
| **Negative regulator of tumour progression** | **Stage** | **Reference** |
| TIMP3<sup>a,b</sup> | HGPIN, primary and metastatic | Yamanaka <i>et al.</i> (2003), Jeronimo <i>et al.</i> (2004c), Yegnasubramanian <i>et al.</i> (2004) |
| S100A6 | Primary and metastatic | Rehman <i>et al.</i> (2005) |
| **Cell adhesion** | **Stage** | **Reference** |
| E-cad/CDH-1<sup>a</sup> | All stages, increasing frequency with disease progression (>50% of metastatic tumours) | Li <i>et al.</i> (2001), Maruyama <i>et al.</i> (2002), Woodson <i>et al.</i> (2003), (2004b), Kang <i>et al.</i> (2004), Singal <i>et al.</i> (2004a), Yegnasubramanian <i>et al.</i> (2004) |
| TSLC1/BL2/IGSF4 | Primary | Sathyanarayanan <i>et al.</i> (2003) |
| LAM-A3, -B3 and -C3 | Primary and metastatic | |
| **Cell growth/proliferation** | **Stage** | **Reference** |
| NEP<sup>c</sup> | Primary | Usmani <i>et al.</i> (2000), Osman <i>et al.</i> (2004) |
| CDH13 | Primary and metastatic | Maruyama <i>et al.</i> (2002) |
| **Apoptosis** | **Stage** | **Reference** |
| FHIT | Primary and metastatic | Maruyama <i>et al.</i> (2002) |
| DAPK | Primary and metastatic | Maruyama <i>et al.</i> (2002), Yamanaka <i>et al.</i> (2003), Kang <i>et al.</i> (2004) |
| DcR2/TRAIL-R4 | NA | Shivapurkar <i>et al.</i> (2004) |
| **Cell-cycle regulation** | **Stage** | **Reference** |
| Cyclin D2 | Primary | Padar <i>et al.</i> (2003) |
| p27/KIP1 | Primary, metastatic | Kibel <i>et al.</i> (2001), Konishi <i>et al.</i> (2002a) |
| p14<sup>a</sup> | Primary | Kang <i>et al.</i> (2004) |
| p16/CDKN2/INK4A<sup>a,b</sup> | HGPIN, primary and metastatic | Jarrard <i>et al.</i> (1997), Konishi <i>et al.</i> (2002b), Maruyama <i>et al.</i> (2002), Jeronimo <i>et al.</i> (2004c), Kang <i>et al.</i> (2004), Yegnasubramanian <i>et al.</i> (2004) |
| **Angiogenesis** | **Stage** | **Reference** |
| THBS1 | Primary | Kang <i>et al.</i> (2004) |
| **Steroid hormone receptors** | **Stage** | **Reference** |
| **DNA repair** | **Stage** | **Reference** |
| MGMT<sup>b</sup> | HGPIN, primary and metastatic | Konishi <i>et al.</i> (2002a), Maruyama <i>et al.</i> (2002), Yamanaka <i>et al.</i> (2003), Jeronimo <i>et al.</i> (2004c), Kang <i>et al.</i> (2004), Yegnasubramanian <i>et al.</i> (2004) |
| **Transcriptional regulation** | **Stage** | **Reference** |

NA, not available. Primary prostate cancer denotes both organ-confined and advanced tumours that extend beyond the prostatic capsule.

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<sup>b</sup>Methylation of these genes occurs at similar frequencies in malignant and nonmalignant tissue, although often at exonic CpG sites and at lower densities in non-tumour tissue.

<sup>c</sup>Methylation has only been assessed in samples with loss of gene expression.

intraepithelial neoplasia (HGPIN) is a widely accepted CaP precursor (Steiner 2003), and proliferative inflammatory atrophy (PIA) has recently been proposed as a preinvasive lesion (De Marzo <i>et al.</i> 1999a), a theory supported by both morphological and molecular data (Nakayama <i>et al.</i> 2003, Putzi & De Marzo 2000).
synthase 2 (PTGS2) appears to increase progressively from normal prostate to HGPIN and throughout disease progression (Jeronimo et al. 2004a, 2004c, Kang et al. 2004, Maruyama et al. 2002, Yamanaka et al. 2003). These findings support a model of an epigenetic catastrophe, with multiple CpG islands becoming hypermethylated very early in the progression of CaP, probably between the occurrence of HGPIN and the development of organ-confined, well-differentiated CaP (Yegnasubramanian et al. 2004). The progressive accumulation of cells that carry these epigenetic alterations (conceivably resulting in a growth or survival advantage) might promote the acquisition of invasive and subsequent metastatic abilities, which could explain why cells harbouring these epigenetic alterations become the dominant population in the invasive carcinoma (Jeronimo et al. 2004c).

**Hypermethylation of GSTP1: as good as it gets?**

Currently, the best-characterised gene that is methylated in CaP is GSTP1. Methylation is highly tumour-specific but also prevalent in HGPIN lesions, which makes GSTP1 an attractive early detection biomarker.

Hypermethylation and inactivation of genes involved in DNA repair, such as GSTP1, may serve as initiating genome lesions for tumour development by increasing susceptibility to carcinogens, thus predisposing to further mutations and DNA damage (Fig. 1; Lee et al. 1994). GSTP1 functions in the conjugation and detoxification of potential carcinogens (Hayes & Pulford 1995) and has been demonstrated to have ‘caretaker’ activities (Adler et al. 1999, Xia et al. 1996). Promoter hypermethylation accompanied by loss of GSTP1 is one of the earliest and most common somatic genome alterations in CaP. It is widely reported in 75–100% of CaPs and in up to 70% of HGPIN lesions (Brooks et al. 1998, Kang et al. 2004, Lin et al. 2001a, 2001b, Nakayama et al. 2003, Woodson et al. 2004b). This range of detection may result from the multiple discrepancies discussed earlier. GSTP1 methylation has also recently been reported in 6% of PIA lesions from CaP patients.

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**Figure 1** An overview of some of the pathways affected by hypermethylation (oval containing 5 mC) in the pathogenesis of CaP. (1) Conversion of testosterone to the more potent dihydrotestosterone (DHT) permits transactivation of the androgen receptor (AR). This facilitates transcriptional regulation of target genes, which leads to enhanced cellular proliferation and reduced apoptosis. Hypermethylation-associated AR gene inactivation in a proportion of hormone-refractory CaPs may confer a growth advantage through alternative pathways that are capable of ‘bypassing’ the need for AR (reviewed by Feldman & Feldman 2001). (2) The functions of cell adhesion proteins such as E-cad, CD44 and TSLC1 become abrogated by promoter hypermethylation during invasion and metastases of CaP. (3) Hypermethylation-driven loss of function of key cell-cycle regulators such as p16, p27 and RASSF1A, allows uncontrolled proliferation. (4) Promoter methylation (of intracellular detoxification enzyme GSTP1) and global hypomethylation (Hypo M) promote genome damage. PI3K, phosphoinositide 3-kinase; IGF1, insulin-like growth factor I; IGFIR, insulin-like growth factor I receptor.
GSTP1, P16, MGMT, ARF Urine 100 87 Hoque et al
GSTP1, histological review Biopsy 100 79 Harden
RAR

Benign prostate) and can correctly identify 77–98% of tumours (Jeronimo et al. 2004c, Bastian et al. 2005a). An extensive study that quantified methylation of 16 different genes described a four-locus panel of GSTP1, APC, PTGS2 and MDR1 that could distinguish primary CaP from benign prostate tissue with 92% specificity and a sensitivity approaching 100% (Yegnasubramanian et al. 2004).

Hypermethylation in biopsies to aid diagnosis

As stated above, one of the problems with the diagnosis of CaP from histological review of biopsy specimens is the false negative rate of ultrasound-guided core-needle biopsy. A number of studies have examined the ability of GSTP1 methylation to improve the sensitivity of standard histology for CaP detection in needle biopsies (Table 3). QMSP analysis of GSTP1 in conjunction with histological review of needle biopsy specimens has been demonstrated to improve the sensitivity of tumour detection in pathologically reviewed, resected prostates by 11%, without compromising on specificity (Harden et al. 2003). To date, there is only one published report that has examined the efficacy of a multi-gene methylation panel to aid CaP diagnosis in biopsy cores (Tokumaru et al. 2004). While the panel of four loci (GSTP1, APC, TIG1, RARβ2) improved the sensitivity of CaP diagnosis by 33% over histology alone, the authors acknowledged that their use of frozen biopsy sections are more

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Sample Type</th>
<th>Specificity %</th>
<th>Sensitivity %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1, histological review</td>
<td>Ejaculate</td>
<td>100</td>
<td>50</td>
<td>Goessler et al. 2000, 2001, Suh et al. 2000</td>
</tr>
<tr>
<td>RARβ2, GSTP1, TIG1, APC</td>
<td>Biopsy</td>
<td>100</td>
<td>79</td>
<td>Harden et al. 2003</td>
</tr>
<tr>
<td>GSTP1, P16, MGMT, ARF</td>
<td>Urine</td>
<td>100</td>
<td>87</td>
<td>Tokumaru et al. 2004</td>
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<td></td>
<td></td>
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<td>Hoque et al. 2005</td>
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</table>

aOnly Sidransky’s group (Cairns et al. 2001, Harden et al. 2003, Tokumaru et al. 2004) performed surgical pathologic review of resected prostates after biopsy to confirm the number of patients with and without prostate cancer. Other studies relied on the efficacy of biopsy alone in detecting prostate cancer. Since biopsy may miss microscopic foci of tumour, we believe the specificity of methylation in bodily fluids (plasma, serum, urine) and the biopsy itself cannot be reliably determined in these men since they cannot be considered cancer-free at that point in time.

bThe large range in detections may result from a number of factors such as quantitative versus nonquantitative methylation assays, the performance (or not) of prostatic massage before voiding urine, which may enrich the urine with prostatic cells, and the assessment of patients at different stages of disease.
difficult to review histologically than standard paraffin sections. Although in its infancy, these reports suggest that with further validation of markers and standardisation of techniques, it may be possible to significantly increase the sensitivity of detecting low-volume disease and to stratify patients with negative biopsy results into low-risk (surveillance) and high-risk (repeat biopsy) sub-groups.

**Hypermethylation in bodily fluids**

Efforts are also underway to develop non-invasive methods of quantifying methylation of genes in bodily fluids such as urine sediments and in extra-cellular DNA present in peripheral blood plasma and serum (Cairns *et al.* 2001, Goessl *et al.* 2000, Jeronimo *et al.* 2001; Table 3). Since alterations in DNA methylation are among the earliest and most common events in tumourigenesis, monitoring methylation patterns via bodily fluids in men at risk for harbouring CaP (elevated PSA, detection of HGPIN on serial biopsy) may detect disease that has been missed by needle biopsy.

More than 75% of tumours originate in the peripheral zone of the prostate gland, which surrounds the urethra. It is therefore conceivable that cellular debris and DNA shed into the urethra by the tumour is detectable in urine. Conversely, high levels of tumour DNA are reported in plasma and serum (Boddy *et al.* 2005). This could indicate that tumour DNA is predominantly released into the circulation rather than into the prostate ductal system.

The tumour specificity of both sources is excellent (with identical methylation patterns reported in CaP DNA), but the sensitivity of detection of localized CaP by GSTP1 methylation alone ranges from 19 to 30% in urine sediment (Cairns *et al.* 2001, Jeronimo *et al.* 2002) and from 12 to 72% in blood plasma/serum (Goessl *et al.* 2000, Jeronimo *et al.* 2002, Bastian *et al.* 2005b). The detection rate reportedly improves to >70% from both sources in advanced disease (Goessl *et al.* 2000, 2001, Bastian *et al.* 2005b). Although this appears to compromise the feasibility of employing these clinical specimens for early cancer detection, urine has an advantage in that the prostate can be physically manipulated to secrete a much higher influx of cells into the urethra. TRUS-biopsy and prostatic massage prior to urine collection have been shown to significantly increase the rate of GSTP1 methylation detection in urine to 58 and 73% respectively (Goessl *et al.* 2001, Gonzalgo *et al.* 2003).

However, cancers of the bladder and kidney also contribute cellular DNA to urine sediment. Therefore, detection of CaP-specific DNA by methylation would require a panel of carefully selected methylation markers to both detect and discriminate among a variety of urological malignancies. A recent analysis of multiple loci (GSTP1, ARF, P16, MGMT) simultaneously, reported that methylation of at least one of the four genes in urine sediments was able to identify 87% of CaP patients from controls (no evidence of cancer) with 100% specificity (Hoque *et al.* 2005). Together, these findings suggest that urine specimens may hold promise as an adjunct screening tool to PSA. Combined biochemical and epigenetic tests could help to reduce the number of unnecessary biopsies being carried out in men with borderline PSA levels. However, larger sample sizes with long-term follow up are imperative. Also, the impact of prostatic massage on the detection rate of methylation in urine needs to be better defined and, in examining the specificity for CaP, other genitourinary malignancies should be included.

**Promoter hypermethylation as a prognostic indicator for CaP**

Metastasis is the major cause of CaP-related death. Methylation biomarkers that could indicate the future development of metastasis in men with primary disease would be of major clinical use. While gland-confined disease is potentially curable with surgery or radiotherapy, these treatments do not suffice for locally advanced and metastatic CaP. Because of the androgen dependence of prostate cells for their proliferation and survival, androgen-ablation therapy is the primary treatment for these tumours. However, a positive response to this treatment is short-lived (1–2 years), and the cancer subsequently manifests as a lethal, hormone-refractory (HR) disease. Many different mechanisms are believed to contribute to the HR phenotype, synonymous with advanced CaP (Feldman & Feldman 2001). Promoter hypermethylation may represent one such mechanism; some of the most noteworthy examples are discussed below.

**Steroid hormone receptors**

CaP is a hormonal malignancy and methylation of several members of the steroid hormone receptor family (androgen receptor (AR), estrogen receptor, RARβ2) has been implicated in disease progression.
Hypermethylation of specific consensus regions within the AR promoter may represent one mechanism responsible for HR disease. Most CaPs express the AR to some extent, but the level of heterogeneity increases with disease progression (Magi-Galluzzi et al. 1997). Reduced AR expression has been associated with a poor response to hormone ablation therapy (Sadi & Barrack 1993), with 20–30% of HR tumours characterized by extensive loss of AR expression (Hobisch et al. 1996, Magi-Galluzzi et al. 1997), which may occur at the transcriptional level, independent of mutational or deletional mechanisms (Tilley et al. 1990). Methylation of the AR promoter-CpG island has been intensely studied and illustrates that targeted CpG sites may be crucial in effecting methylation-mediated transcriptional silencing (Fig. 2A). Methylation at two hotspots within core promoter regions is associated with AR inactivation in vitro (in metastatic CaP cell lines DU145 and PC-3) and in vivo, in up to 20% of late-stage primary tumours and up to 28% of HR tumours (Jarrard et al. 1998, Kinoshita et al. 2000, Nakayama et al. 2000, Sasaki et al. 2002).

Metastasis suppressors

Functional data strongly argue that there are specific genes that control metastasis, defined by their ability to suppress metastasis in vitro, without blocking tumourigenicity (Welch & Hunter 2003). Downregulation or complete loss of metastasis suppressor genes E-cadherin (E-cad) and CD44 is repeatedly correlated with clinical progression; increased histological grade, advanced clinical stage, presence and extent of metastases and an unfavourable outcome (Umbas et al. 1992, Kallakury et al. 1996, Noordzij et al. 1997, Richmond et al. 1997). Both function in cell adhesion, thereby potentially influencing the ability of secondary tumour cells to invade tissues and adhere to the endothelium (Gao et al. 1997, Kauffman et al. 2003). Frequent promoter hypermethylation and subsequent silencing of both genes occurs in CaP cell lines in vitro and is associated with tumour progression in vivo (Fig. 1; Graff et al. 1995, Lou et al. 1999, Verkaik et al. 1999, Li et al. 2001, Kang et al. 2004, Woodson et al. 2004a). Transient downregulation of E-cad appears to promote metastasis (De Marzo et al. 1999b), with distinct methylation patterns occurring between different pathological grades of CaP (Fig. 2B; Li et al. 2001). In this case, the evaluation of different CpG sites could be exploited for the design of prognostic methylation biomarker assays.

Neural endopeptidase 24.11 (NEP; CD10) is a cell-surface peptidase that inactivates neuropeptides, which have been implicated in progression to HR CaP (Sumitomo et al. 2001). Overexpression of NEP in HR CaP cells significantly inhibits their growth (Dai et al. 2001), while loss of NEP activity stimulates CaP cell proliferation and migration (Fig. 1; Sumitomo et al. 2001). Transcriptionally activated by androgens, it demonstrates reduced expression in androgen independent (AI) cells in vitro (Papandreou et al. 1998). Promoter hypermethylation also contributes to the inactivation of NEP in AI CaP cell lines (PC-3 and DU145; Usmani et al. 2000) and in over 70% of primary CaPs (Osman et al. 2004).

Notably, CpG island hypermethylation patterns in CaP metastases have been described as highly similar to those found in primary cancers. Differences between cases are greater than between anatomical sites of metastasis, indicating that patterns of hypermethylation are maintained in a clonal manner even through the process of metastatic progression (Bastian et al. 2005a, Yegnasubramanian et al. 2004).

Clinicopathologic correlations

It is thought that approximately 30% of men diagnosed with clinically localised disease will undergo either biochemical (elevation of PSA) and/or clinical recurrence and progress to metastatic androgen-independent disease (Pound et al. 1999, Blute et al.
Current prognostic indicators include Gleason score (a measure of cancer cell differentiation) ≥7, pathological stage, pre-operative serum PSA level ≥8 ng/ml, and PSA doubling time (Partin et al. 1997). Correlations between specific gene hypermethylation and clinicopathologic features suggestive of aggressive disease characteristics indicate that these genes may have prognostic potential. Such molecular markers may help to identify men who will undergo recurrence, so that they can be targeted for more aggressive therapy. Several studies have now shown that the ‘methylation index’, defined as the ratio of methylated genes to the total number of genes analysed, correlates with clinicopathologic indicators of poor prognosis (Maruyama et al. 2002, Kang et al. 2004, Bastian et al. 2005a). Correlations have also been made for some individual genes, such as LAMA3 (Sathyanarayana et al. 2003), HIN-1 (Shigematsu et al. 2005), CDH13 (Maruyama et al. 2002), cyclin D2 (Padar et al. 2003) and TIG1 (Zhang et al. 2004), but these need to be confirmed in more robust studies, since results for several genes (APC, CD44, E-cad, MDR1, RARβ2 and RASSF1A) are inconsistent between individual reports (Maruyama et al. 2002, Yamanaka et al. 2003, Enokida et al. 2004, Jeronimo et al. 2004b, 2004c, Kang et al. 2004). However, the methylation frequencies of E-cad as well as genes such as PTGS2 and RUNX3 have been correlated with a higher risk of PSA recurrence independently of Gleason score or pathological stage (Maruyama et al. 2002, Kang et al. 2004, Yegnasubramanian et al. 2004). In addition, quantitative studies have shown a relationship between increasing methylation levels of certain genes (GSTP1, APC, RARβ2 and endothelian receptor type B [EDNRB]) in tumours with advancing pathological stage and/or high Gleason score (Jeronimo et al. 2004c, Yegnasubramanian et al. 2004).

The role of hypomethylation in CaP

Perhaps surprisingly, DNA hypomethylation was actually identified in human tumours before DNA hypermethylation (Feinberg & Vogelstein 1983). Studies in the early 1980s suggested that cancer genomes (especially those of metastases) were hypomethylated when compared with normal cells (Bedford & van Helden 1987, Gama-Sosa et al. 1983). There are a number of ways in which genome-wide de novo demethylation could promote carcinogenesis. Hypomethylation of repetitive sequences, such as LINE-1 retrotransposons, causes their transcriptional activation, which can disrupt the expression of adjacent genes (Chalitchagorn et al. 2004). DNA hypomethylation leads to elevated mutation rates by destabilizing the genome and promoting loss of heterozygosity in regions containing tumour suppressors. Through its decondensing effect on chromatin structure, it might also facilitate chromosomal instability, particularly chromosome breaks, deletions, amplification and illegitimate recombination (Fig. 1; Ehrlich 2002).

Until recently, extensive global hypomethylation had only been observed in HR and metastatic CaPs (Bedford & van Helden 1987, Florl et al. 2004, Santourlidis et al. 1999, Schulz et al. 2002). Now, with the availability of more refined techniques (that quantify methylation in all genomic regions not only within repetitive elements), significant levels of hypomethylation have been demonstrated in 97% of primary CaPs (Brothman et al. 2005), implying a major role for hypomethylation at all stages of tumourigenesis.
A number of recent studies suggest that de novo hypomethylation may also target single-copy gene promoter-CpG islands, leading to proto-oncogene activation. Hypomethylation and abnormal expression of bone morphogenetic protein-6 in metastatic CaP is speculated to play a role in bone-forming skeletal metastasis (Tamada et al. 2001). Heparanase and urokinase plasminogen activator (uPA) promote tumour growth, invasion and metastasis through degradation of components of the extracellular matrix (Vassalli & Pepper 1994, Hulett et al. 1999). Increased heparanase expression in CaP has been demonstrated to occur through coconjugate promoter hypomethylation and upregulation of the transcription factor EGR1 (Ogishima et al. 2005). Promoter methylation is the primary molecular mechanism that reversibly regulates uPA expression at different stages of CaP progression, inactivating uPA in the normal prostate cell line PrEC and in the hormone-sensitive cell line LNCaP (Guo et al. 2002, Pakneshan et al. 2003). Conversely, the gene is hypomethylated and expressed in HR cell line PC-3 and in the plasma of metastatic CaP patients (Hienert et al. 1988). Treatment with 5-aza-C induces uPA expression in LNCaP cells and results in an increase in their invasive capacity (Pakneshan et al. 2003). This loss in the balanced activity between the methylating (DNA methyltransferases) and demethylating (demethylase) enzymes allows a switch from hypermethylated to hypomethylated uPA in the progression of CaP (Guo et al. 2002).

Taken together, these findings suggest that hypomethylation could prove a significant force in prostate carcinogenesis. Whether DNA hypomethylation will yield any potential biomarkers for CaP remains to be answered. To date, few associations have been made with clinicopathologic features of poor prognosis, such as either tumour grade or Tumour node metastases (TNM) stage (Schulz et al. 2002, Liu et al. 2005).

The interrelationship of hypo- and hypermethylation in malignancy is interesting to consider. Global hypomethylation and targeted hypermethylation together confer a selective advantage upon cancer cells by targeting different sets of genes with opposing roles in cellular transformation (Szyf et al. 2004). Promoter hypermethylation of GSTP1 has been reported to precede genome-wide hypomethylation in the development of prostate carcinoma (Santourlidis et al. 1999). LINE-1 hypomethylation has been associated with hypermethylation of several genes (GSTP1, RARβ2 and APC; Flor et al. 2004). The balance between hyper- and hypomethylation may be important in predicting clinical outcomes such as disease recurrence (Brothman et al. 2005).

The potential for DNA methylation in novel therapeutic strategies

The potential to reverse DNA methylation is an attractive therapeutic avenue, since epigenetic modifications effect a change in gene expression without altering gene function. Two closely related drugs, 5-aza-C and 5-aza-2’-deoxycytidin (5-aza-dC; Decitabine), are used experimentally to inhibit DNA methylation in vitro and have been demonstrated to re-activate numerous methylation-silenced genes, such as GSTP1 (Lin et al. 2001b) and RARβ2 (Nakayama et al. 2001). 5-aza-C and 5-aza-dC are cytosine analogues that become incorporated into DNA and trap DNMT1 during replication, leading to synthesis of nascent DNA in the absence of DNMT activity, resulting in DNA demethylation (Taylor & Jones 1982, Friedman 1985). The administration of these drugs causes multiple changes as a consequence of demethylation, including activation of silenced genes, decondensation of chromatin and induction of cellular differentiation (Goffin & Eisenhauer 2002).

HDAC inhibitors, such as trichostatin A and butyrate, have been used synergistically with demethylating agents to activate expression of methylated genes (Cameron et al. 1999). Co-treatment of AR-negative cell line DU145 with 5-aza-C and trichostatin A is most effective in restoring functional expression of AR and its downstream targets such as PSA, compared with either agent alone (Jarrard et al. 1998, Nakayama et al. 2000). Furthermore, treatment with methylation inhibitor 5,6-dihydro-5’-aza-cytidine increased the sensitivity of DU145 to anti-androgen hydroxyflutamide (Izbicka et al. 1999).

However, the clinical utility of demethylating drugs in cancer treatment has been limited by their toxicity. This is mediated primarily through covalent binding of DNMT to 5-aza-C-substituted DNA rather than genomic DNA demethylation (Juttermann et al. 1994). To date, clinical trials of these agents in haematological malignancies have proved the most successful (Goffin & Eisenhauer 2002). Decitabine is undergoing phase II clinical trials for the treatment of HR metastatic CaP (Thibault et al. 1998). Serious side effects include myelosuppression and the promotion of malignant transformation of genes, through induction of global DNA
hypomethylation (Denda et al. 1985, Jackson-Grusby et al. 1997). This has prompted development of non-nucleoside analogue DNMT inhibitors, such as procainamide, and the use of DNMT antisense approaches that protect the genome from global hypomethylation (Bigey et al. 1999, MacLeod & Szyf 1995). Procainamide has been demonstrated to reverse GSTP1 methylation and restore its expression in LNCaP cell lines propagated both in vitro and in vivo as xenograft tumours in mice (Lin et al. 2001a). Antisense oligonucleotides, directed against DNMT1 mRNA, reduced DNMT1 activity, induced demethylation and re-expression of p16 in human tumour cells (Fournel et al. 1999) and inhibited tumour growth and induced demethylation in mouse models (Ramchandani et al. 1997).

Since promoter hypermethylation often occurs during the early stages of many human cancers, targeting the epigenome is a potentially widely applicable chemopreventative strategy. Treatment with 5-aza-dC has been shown to reduce the appearance of intestinal polyps (preinvasive lesions for colorectal carcinoma) in APC− and APC+ mice (Laird et al. 1995). In CaP, the apparent critical role for GSTP1 inactivation in the earliest steps of carcinogenesis could in future provide attractive opportunities for CaP-prevention strategies, including restoration of GSTP1 function, compensation for inadequate GSTP1 activity and attenuation of genome-damaging stresses (Nelson et al. 2001).

Future directions

Clearly, one of the most important and challenging areas of CaP management is the need to discriminate between latent, occult disease that may never progress and cancers that are likely to grow and metastasize rapidly. Age is one of the strongest risk factors for CaP, with the majority of tumours diagnosed in men over the age of 65 and the risk increasing steeply thereafter (Jemal et al. 2005b). Examinations of non-neoplastic prostate samples show methylation of multiple genes, albeit at lower frequencies than in tumour. But methylation of certain loci such as the calcium-binding protein S100A2 gene has been demonstrated in a high proportion of both malignant and BPH samples (Jeronimo et al. 2004c, Rehman et al. 2005). While these results could indicate that hypermethylation may precede morphological changes, equally they may argue for age-associated hypermethylation of certain genes (S100A2 and RASSF1A) in the prostate, like that suggested for other organs such as the colon (Toyota & Issa 1999). Investigations into the prevalence of methylation effects in occult CaPs of younger men may shed insight into the cause or consequence of tumour-related methylation.

Geographical and ethnic correlations

CaP is characterised by marked geographical and ethnic variation. Substantial differences in incidence and mortality exist between US African-Americans and Caucasians, whereas Asian men have the lowest risk (Jemal et al. 2005a). Although, the molecular causes underlying racial differences in CaP incidence and clinical behaviour have not been well characterised, preliminary findings suggest that differences in gene hypermethylation could play a role. A 1.7-fold higher frequency of CD44 methylation was found in African-American men compared with Caucasian-American men with CaP (Woodson et al. 2003). Hypermethylation of CD44 and GSTP1 have independently been correlated with poor pathologic findings in Asian, but not American men (Kito et al. 2001, Woodson et al. 2003, Enokida et al. 2005).

Dietary correlations

Despite the prevalence of DNA methylation in human carcinogenesis, causes of aberrant methylation in neoplasia are still not understood. Interestingly, many factors that may influence DNA methylation have been proposed as aetiologic factors for CaP such as genetic, dietary and environmental factors as well as infectious agents. For example, a considerable number of epidemiological and animal studies support a role for dietary factors including folate, selenium, phytoestrogens and polyphenolics in influencing changes in methylation. The relationship between diet and methylation is important to study because diet is an easily modifiable risk factor.

Folate is involved in the synthesis and repair of DNA and in regenerating the universal methyl donor (S-adenosylmethionine). Folate deficiency occurs in human preneoplastic cells and has been associated with increased cancer risk, promoter hypermethylation, genome-wide hypomethylation and DNA lesions (Ma et al. 1997, Pogribny et al. 1997, Beilby et al. 2004). As methyltransferase DNMT1 binds damaged DNA with higher affinity than its cognate hemimethylated CpG sites, available enzyme is sequestered away from replicating DNA, thus promoting demethylation (Yang et al. 1995). Folate deficiency interferes with the methylation
balance by reducing the availability of both a methyl donor and a methyltransferase enzyme.

Selenium, vitamin E and phytoestrogens (e.g. genistein) are recognized as having potential anti-cancer properties (Clark et al. 1998, Heinonen et al. 1998, Mentor-Marcel et al. 2001) and are being investigated as preventative agents against CaP (Hoque et al. 2001). High concentrations of selenium reduce DNMT1 activity and expression in vitro, while selenium deficiency has the reverse effect (Fiala et al. 1998, Davis & Uthus 2004). Consumption of phytoestrogens has been attributed with protecting the methylation profile of prostate tissue (Day et al. 2002) and causing promoter hypermethylation of the ras oncogene in pancreatic cancer (Lyn-Cook et al. 1995).

Gene–nutrient interactions should also be considered when examining the effect of specific dietary elements on DNA methylation. For example, polymorphisms C677T and A1298C in the methyl metabolism gene methylene-tetrahydrofolate reductase (MTHFR) lower its enzymatic activity and may modulate the effect of dietary folate on DNA methylation and cancer susceptibility (Heijmans et al. 2003). Although there is little evidence to support an altered risk in CaP, stronger associations have been shown for higher tumour grade and more advanced disease (Kimura et al. 2000, Cicek et al. 2004, Singal et al. 2004b, Van Guelpen et al. 2006).

**Therapeutic potential**

Possibly the greatest challenge in CaP therapy lies in the effective treatment of HR metastatic disease. To date, only a limited number of preliminary studies targeting the CaP epigenome have been reported. If hypomethylation plays a causal role in promoting metastasis, could inhibition of DNA demethylases effect a reduction in metastasis? Furthermore, if global hypomethylation coordinates the expression of multiple genes, then prevention of this process would be preferable to current approaches targeting specific proteins. A number of recombinant strategies to induce hypermethylation and silencing of prometastatic genes in cancer have been recently reviewed (Szyf et al. 2004). Further laboratory studies and clinical trials are needed to fully establish the therapeutic efficacy of methylation-modifying drugs and their use in combination with HDAC inhibitors and conventional chemotherapeutic agents for CaPs that display a methylator phenotype.

**Conclusion**

Exploiting DNA methylation offers several exciting and promising opportunities for the management of CaP (Fig. 3). Promoter methylation is a frequent, early event and accumulates during multi-step prostatic carcinogenesis. As such, specific targets of hypermethylation in CaP have been and continue to be defined. The development of these targets as methylation biomarkers for CaP diagnosis and prognosis could contribute to the optimal identification and treatment of this disease. With the advent of new, highly sensitive quantification tools, the list of potential CaP markers grows daily. Sifting through these genes to translate research findings into clinically relevant tests will require rigorous analysis.
and standardisation. The prevalence of methylation of multiple targets often differs significantly between different studies. Many of these discrepancies certainly arise from different methodologies used, and the use or not of microdissection techniques that allow for the isolation and analysis of pure populations of cells. However, the results described could potentially reflect a true difference between different patient groups and/or different populations (which are affected by significant differences in risk). These dissimilarities emphasize the need for standardised methodologies if DNA methylation is intended as a molecular diagnostic tool.

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