

Abnormal DNA methylation, epigenetics, and prostate cancer

William G. Nelson, Srinivasan Yegnasubramanian, Agoston T. Agoston, Patrick J. Bastian, Byron H. Lee, Masashi Nakayama, Angelo M. De Marzo

Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Brady Urological Institute, Baltimore, MD 21231

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1. ABSTRACT

Human prostatic carcinogenesis is characterized by the accumulation of both genetic and epigenetic alterations. The epigenetic changes appear earlier and more consistently, and because the DNA sequence remains intact, may be therapeutically reversible. The mechanism(s) by which epigenetic changes appear during the pathogenesis of prostate cancer have not been established. Nonetheless, new methods for the detection of abnormal DNA methylation, a molecular biomarker of epigenetic alterations, are poised to provide clinical tests potentially useful for prostate cancer detection and diagnosis. In addition, new drugs targeting DNA methyltransferases and other enzymes involved in the maintenance of chromatin structure have been introduced into clinical trials for the treatment of advanced prostate cancers. If sufficiently safe strategies for chromatin modulation can be discovered and developed, epigenetic alterations may become rational targets for both prostate cancer prevention and prostate cancer treatment.

2. INTRODUCTION

Prostate cancer cells, like other cancer cells, characteristically carry a plethora of somatic genome defects that contribute to a phenotype characterized by inappropriate proliferation, evasion of apoptosis, tissue invasion, induction of angiogenesis, escape from immune surveillance, and metastasis (1). Some of the genome defects are genetic changes (changes in DNA sequence), such as gene mutations, deletions, amplifications, and translocations. Other genome defects are epigenetic alterations, including changes in cytosine methylation patterns and chromatin structure (2). In the pathogenesis of human prostate cancer, somatic epigenetic alterations appear earlier than genetic changes, as well as more commonly and consistently. Several of the genes silenced by epigenetic alterations have been identified, providing new molecular biomarkers of prostate cancer and new mechanistic clues into prostate cancer etiology. However, the mechanism by which epigenetic alterations accumulate during prostatic carcinogenesis has not been established. A

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more thorough understanding of the acquisition of epigenetic alterations during prostatic carcinogenesis may provide new insights into how prostate cancer can be better treated and/or prevented.

3. DNA HYPERMETHYLATION, HETEROCHROMATIN, AND EPIGENETIC GENE SILENCING

The self-complementary nucleotide sequence CpG is under-represented in mammalian genomes and frequently contains a modified cytosine base ($^{5\text{-meC}}$), carrying a methyl group at the 5 position. Unmethylated CpG dinucleotides, clustered into ~ 1 kb regions encompassing the transcription start sites of many genes, have been termed "CpG islands" (3). In this state, chromatin at these CpG island regions can be molded into active conformations by transcriptional *trans*-activators, recruiting histone acetyltransferases and histone methyltransferases that can facilitate the loading of RNA polymerases onto gene promoters. However, when the CpG island sequences contain $^{5\text{-meC}}$, especially if $^{5\text{-meC}}$ is present at high densities, the chromatin structure poses a significant barrier to transcription, with the recruitment of histone deacetylases in place of histone acetyltransferases, etc. When condensed in this way, the chromatin resembles the facultative heterochromatin characteristic of inactive X-chromosomes in female cells, with gene promoter DNA tightly wound around histone octamers into nucleosomes in a manner resistant to RNA polymerase loading. All cancer cells, including prostate cancer cells, appear to commonly exploit this epigenetic means of gene repression, characterized by transcriptionally inactive chromatin and CpG island hypermethylation, in the acquisition and maintenance of the neoplastic phenotype (2,4,5). Genes somatically "silenced" in this way are thus analogous to genes that have functionally inactivated by somatic deletion or mutation (2). Furthermore, like genetic defects, the abnormal CpG dinucleotide patterns in cancer cells can be propagated and maintained throughout DNA replication and cell division. As of yet, whether the establishment of transcriptionally silent heterochromatin at the loci of critical cancer genes proceeds via initial DNA modification versus initial histone modification, or via a pathway initiated by small interfering RNA species, has not been resolved. However, interactions between DNA methylation and histone modification likely ensure maintenance of repressive chromatin structures through genome replication and cell division.

Polycomb group proteins are known to function in maintaining selective gene repression during development. Enhancer of zeste homolog 2 (EZH2), a histone H3-K27 methyltransferase component of polycomb group complexes, has been reported to be highly expressed in metastatic prostate cancers (6). Furthermore, siRNA-mediated reduction EZH2 levels in prostate cancer cells resulted in an inhibition of cell proliferation while forced EZH2 over-expression triggered repression of a specific set of genes (6). EZH2 likely acts to repress genes when recruited, along with other polycomb group proteins, to specific genome sites by DNA binding factors. At these

sites, creation of histone H3-K27 marks facilitates assembly of repressive chromatin complexes (7). One candidate target gene for EZH2-mediated repression is *DAB2IP*, encoding a GTPase-activating protein that can affect Ras signaling and tumor necrosis factor (TNF)-associated apoptosis. In prostate cancer, the loss of *DAB2IP* expression has been attributed to epigenetic silencing (8). A recent report has implicated EZH2 in *DAB2IP* silencing, showing that forced over-expression of EZH2 in normal prostate cells suppressed *DAB2IP* expression, while siRNA-mediated knock-down of EZH2 in cancer cells increased *DAB2IP* production (9). In the silenced state, the *DAB2IP* promoter region carried histone H3-K27 marks, consistent with EZH2 function (9). Thus, it appears likely that dysregulation of EZH2 expression may be capable of promoting epigenetic gene silencing. Nonetheless, whether EZH2 expression, likely regulated by the pRB-E2F pathway and required for cell replication, merely reflects increased proliferation in prostate cancers or indicates epigenetic gene dysregulation has not been established (10).

Epigenetically silenced genes appear to be repressed via the actions of $^{5\text{-m}}$ CpG-binding domain (MBD) family proteins (11). One of the MBD family proteins, MeCP2, contains a ~ 70 amino acid minimal region that mediates selective binding to DNA containing $^{5\text{-m}}$ CpG (an MBD motif), and a transcriptional repression domain (TRD) that permits interacts with Sin3 and Sin3-bound HDACs to repress transcription (12). By binding to hypermethylated CpG islands in cancer cells, MeCP2 can thus silence critical genes in a manner dependent on HDAC activity, a phenomenon potentially antagonized by HDAC inhibitors such as trichostatin A and others (12). Another MBD family protein, MBD2, which also binds selectively to DNA containing $^{5\text{-m}}$ CpG, is a component of a 1 MD transcription repression complex, MeCP1, endowed with Mi-2/NuRD chromatin remodeling complex components such as MBD3, HDAC1 and HDAC2, histone-binding proteins RbAp46 and RbAp48, the SWI/SNF helicase/ATPase domain-containing protein Mi-2, MTA2, and other polypeptides (13). For this repression complex, MBD2 serves to direct chromatin remodeling activity to CpG islands containing $^{5\text{-m}}$ CpG (13). Of interest, although MeCP2-mediated transcriptional repression can typically be alleviated by treatment with HDAC inhibitors, MeCP1-mediated inhibition of $^{5\text{-m}}$ CpG-containing promoter activity is often not affected by HDAC inhibitor exposure (14). Cells from *Mbd2*^{-/-} mice, as well as human cancer cells treated with siRNA targeting *MBD2* mRNA, are unable to repress transcription from exogenously hypermethylated promoters in transient transfection assays (15,16).

Independent of the mechanism(s) by which abnormal epigenetic gene silencing appears during cancer development, changes in gene function associated with abnormal DNA methylation appear subject to selection for cell growth and/or survival. Luria-Delbruck fluctuation analyses have hinted that CpG island hypermethylation targeting the gene that encodes the p16/INK4a cell cycle regulatory protein can arise spontaneously by some mechanism in a small minority of human mammary

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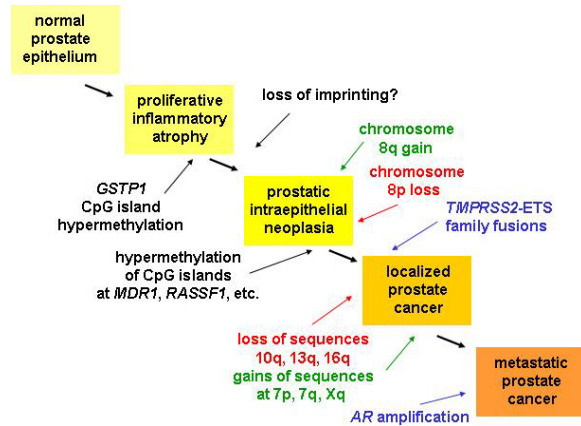


Figure 1. Accumulation of somatic genetic and epigenetic alterations during the pathogenesis of prostate cancer. Epigenetic changes, such as loss of imprinting and CpG island hypermethylation at *GSTP1* and other key genes, arise earlier than genetic changes, including *TMPRSS2-ETS* family fusions, chromosomal gains/losses, and *AR* amplification.

epithelial cells (HMECs) (17). This somatic epigenetic alteration permits the cells to continue proliferating while unaffected HMECs undergo cell senescence (17). The equivalence of epigenetic and genetic alterations in response to selective pressures during cancer development is further demonstrated by HCT-116 colorectal cancer cells, which like some of the HMEC cells described above, lack p16/INK4a function. HCT-116 cells contain one mutant gene encoding p16/INK4a, with a frameshift mutation in the coding sequence, and one wild-type gene, showing marked hypermethylation at the CpG island region (18). Remarkably, CpG island hypermethylation changes are only present at the gene encoding wild-type p16/INK4a and not at the mutant gene (18). Presumably, hypermethylation of the CpG island at the gene encoding a mutant p16/INK4a would not have provided any selective cell growth advantage. Hypomethylation may also be subject to selective pressures: in MCF-7 breast cancer cells, a reduction in CpG island hypermethylation at *MDR1*, encoding the P-glycoprotein drug transporter, provides a survival advantage to in the face of treatment anti-neoplastic drugs, such as doxorubicin and paclitaxel, that are substrates for efflux pumping (19).

4. DNA HYPOMETHYLATION, DEMETHYLATION, AND LOSS OF IMPRINTING

Many cancer cells, despite carrying hypermethylated CpG island sequences at the loci of several key genes, also contain sequences with hypomethylated CpG dinucleotides (20). In principle, DNA hypomethylation in cancer cells might be the result of inadequate maintenance of CpG dinucleotide methylation during DNA replication, of inadequate remethylation of DNA repair patches, or of active demethylation (21,22). Of these mechanisms, the notion that active demethylation might lead to hypomethylation in cancer has been quite controversial. In early development after fertilization,

genomic DNA present in the male pronucleus has been reported to undergo rapid genome-wide demethylation within hours and without any genome replication (23,24). The enzymatic basis for this phenomenon has not been established, though naked DNA microinjected into oocytes has been found to undergo demethylation (25). Szyf and coworkers have argued that MBD2, or an MBD2 variant, might function as a processive DNA demethylase, contributing to DNA hypomethylation in cancer cells (26,27,28,29). Others have challenged this view, finding no demethylase activity associated with MBD2 (30,31). Of interest, male pronucleus demethylation appears to occur normally in fertilized *Mbd2*^{-/-} oocytes, suggesting that some other demethylase, or other demethylation process, may be responsible for epigenetic reprogramming of paternal genomes during development (32). Currently, the best candidate mechanism for active DNA demethylation features the actions of glycosylases that remove ⁵-meC bases from DNA, followed by replacement with unmethylated cytosines (33,34,35). Whether such a mechanism may be responsible for the appearance of hypomethylation in cancer cells has not been ascertained.

One phenomenon associated both with DNA hypomethylation, as well as with DNA hypermethylation, is loss of the fidelity of gene imprinting, a process involving the physiological silencing of certain gene alleles, such as the maternal *IGF2* allele, during development depending on the parental origin (36,37,38,39). In a murine model, loss of *Igf2* imprinting resulted in a doubling of the amount of tumors in *Apc*^{Mim/+} mice (40). Furthermore, mouse embryonic fibroblasts derived from imprint-free embryonic stem cells appear to be resistant to TGFβ, to express reduced levels of p19 and p53, to immortalize spontaneously, and to form tumors *in vivo* (41). Loss of imprinting appears common to many human cancers, including prostate cancer (42,43). CpG dinucleotide hypomethylation has also been proposed to promote cancer development by causing inappropriate gene activation or regulation, increased gene recombination, and/or de-repression of endogenous retrovirus genes (42,44,45). In support of this mechanism, mice carrying defective genes for one of the DNA methyltransferases (DNMTs) needed to maintain genomic CpG methylation patterns genes exhibit both genome-wide under-methylation and a susceptibility to lymphomagenesis (44).

5. DNA METHYLTRANSFERASES AND CANCER DEVELOPMENT

DNA methyltransferases (DNMTs), which can catalyzing the transfer of a methyl group from *S*-adenosyl-methionine to cytosine bases in CpG dinucleotides, are responsible for establishing and maintaining patterns of CpG dinucleotide methylation in the genome through DNA replication and mitosis. Although it is clear that the fidelity of CpG methylation pattern maintenance must be somehow corrupted in cancer cells, the means by which acquired increases and decreases in CpG dinucleotide methylation appear during the development of cancer have not been fully established. One proposed mechanism attributes somatic abnormalities in CpG dinucleotide methylation

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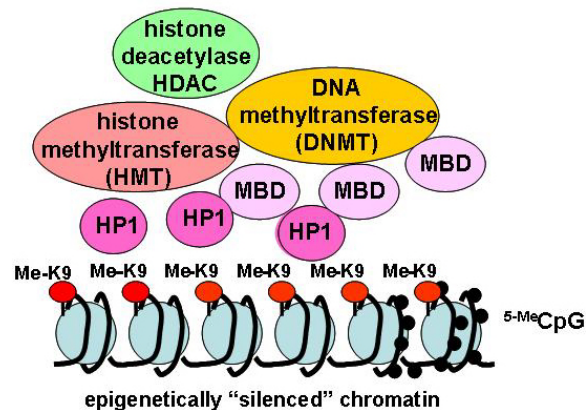


Figure 2. Epigenetic gene silencing involves the assembly and maintenance of a repressive chromatin structure, featuring CpG dinucleotide methylation, specific histone marks (such as loss of acetylation and certain methylation modifications), and binding of ^{5-mc}CpG binding domain proteins (MBDs) and HP1.

directly to inappropriate expression or activity of DNMTs or demethylases (28,46). Another model suggests that DNA methylation changes, particularly those affecting specific genes, are more likely the indirect consequences of gene repression by specific *trans*-repressors (47). A newer idea is that somatic DNA methylation changes may be a product of some sort of chromatin structure modulation pathway such as one involving regulatory RNAs and DNMTs (48,49).

Aberrant DNMT function may contribute both to DNA hypermethylation and hypomethylation during cancer development. There are three known mammalian DNMTs responsible for CpG dinucleotide methylation: DNMT1, with preferential activity on hemi-methylated DNA substrates, and DNMT3a and DNMT3b, both capable of *de novo* CpG dinucleotide methylation on unmethylated DNA substrates (50). Of the three, DNMT1, targeted to the replication fork during the S-phase of the cell cycle, is the enzyme most responsible for maintaining methylation patterns through genome duplication and segregation at mitosis (51). The enzyme also appears targeted to sites of DNA repair, suggesting that the enzyme may also be responsible for maintaining CpG methylation patterns in regions of the genome threatened by DNA damage (22). In contrast, DNMT3a and DNMT3b act as *de novo* DNMTs: during development, mouse embryos carrying disrupted genes for these enzymes are unable to establish appropriate CpG dinucleotide methylation patterns (52). In cancer cells, some sort of cooperation, or functional compensation, between the various DNMTs has been reported (53,54). Although HCT-116 human colon cancer cells carrying targeted disruptions of DNMT1 or DNMT3b genes lose only 20% and 3% of total genomic cytosine methylation levels, respectively, cells with both genes disrupted lose some 95% of the methylated cytosine bases, indicating that DNMT3b can complement DNMT1 in the maintenance of genomic DNA methylation patterns (53,54).

Of all the DNMTs, DNMT1 appears most likely to play a major role in cancer development. The enzyme has been reported to be required for for *c-fos* transformation of rodent fibroblasts *in vitro*, as well as for intestinal polyp development in *Apc^{Min/+}* mice and for tobacco carcinogen-induced murine lung cancer development *in vivo* (55,56,57,58). In addition, both too much, and too little, DNMT1 function have been implicated in the generation of the abnormal DNA methylation patterns typical of cancer cells. Forced over-expression of DNMT1 in normal cells directly causes increases in DNA methylation and epigenetic gene silencing (46,59,60). In contrast, under-production of the enzyme can also result in cancer development, as mice carrying one disrupted *Dnmt1* allele and one hypomorphic *Dnmt1* allele, resulting in 10% of normal DNMT activity, have been reported to exhibit genomic instability and to develop T-cell lymphomas (44,45). Although dysregulated DNMT1 function has been shown to result in abnormalities in DNA methylation patterns, the mechanism(s) by which the enzyme may malfunction during cancer development have remained elusive. Increases in the expression of *Dnmt1*, along with abnormalities in DNA methylation, have been reported for mouse prostate cells carrying disrupted *Rb* genes, linking the pRb-E2F pathway to regulation of DNA methylation (61). However, mRNA encoding DNMT1 does not appear to be commonly over-expressed or under-expressed, when normalized to proliferative activity, in very many human cancer cells (62). Nonetheless, DNMT1 polypeptides levels appear to be extensively regulated via targeted ubiquitin conjugation and destruction by the proteasome, and many cancer cells appear to display marked defects in this DNMT1 degradation pathway, suggesting that DNMT1 polypeptide over-expression may occur even in the absence of increases in *DNMT1* mRNA levels (63).

6. DNA METHYLATION CHANGES IN PROSTATE CANCER

The first gene found to be silenced via somatic CpG island hypermethylation in prostate cancer was *GSTP1*, encoding the π -class glutathione S-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogens (64). This genome change remains the most common somatic genome abnormality of any kind (>90% of cases) reported thus far for prostate cancer, appearing earlier and more frequently than other gene defects, including the recently described fusions between *TMPRSS2* and ETS family genes, that arise during prostate cancer development (65,66). The associated loss of π -class GST function likely sensitizes prostatic epithelial cells to cell and genome damage inflicted by dietary carcinogens and inflammatory oxidants, perhaps explaining the well-documented contribution of diet and lifestyle factor to prostatic carcinogenesis (65,67,68). Mice carrying disrupted *Gstp1/2* genes are more prone to develop skin tumors upon exposure to a topical carcinogen than wild-type mice (69). Provocatively, *GSTP1* CpG island hypermethylation, which is not present in normal prostatic epithelial cells (nor any other normal cells), seems to arise first in proliferative inflammatory atrophy (PIA) lesions,

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the earliest prostate cancer precursors, which are characterized by simultaneous inflammatory epithelial damage and regeneration (70,71,72,73). Epigenetic silencing of *GSTP1* expression persists in prostatic intraepithelial neoplasia (PIN) lesions, later prostate cancer precursors, and in prostatic carcinomas, hinting at some sort of selective growth or survival advantage (68,73,74,75). The recognition that DNA hypermethylation changes characteristic of prostate cancer cells first appear in PIA lesions suggests that chronic or recurrent inflammation may play some role in the *de novo* acquisition of abnormal DNA methylation patterns. Of interest in this regard is a report of interleukin 1 β -triggered silencing of *FMRI* and *HPRT* in cells via a mechanism featuring nitric oxide generation (76). Activated macrophages, expressing high levels of the inducible form of nitric oxide synthetase (iNOS), have been detected near PIA lesions in human prostate tissues.

Since the recognition that the *GSTP1* CpG island was frequently hypermethylated in prostate cancers, more than 40 genes have been reported to be targets of DNA hypermethylation-associated epigenetic gene silencing in prostate cancer cells (5). From all the studies reported thus far, it seems likely that CpG island hypermethylation changes appear in at least two waves, first in prostate cancer precursor lesions, as the genome changes that initiate neoplastic transformation, and then later in transformed cells, as the genome changes that drive malignant progression. For example, in one case series, hypermethylation of CpG islands at *GSTP1*, *APC*, *RASSF1a*, *COX2* and *MDR1* was present in the majority of localized prostate cancers and persistent in the majority of advanced metastatic cancers, while hypermethylation at the *ER α* , *hMLH1*, and *p14/INK4a* CpG islands was rare in primary cancers and more common in metastatic cancer deposits (4). Finally, in a study of men with lethal prostate cancer who underwent autopsies, analysis of CpG island hypermethylation profiles from different metastatic deposits in different anatomic sites from different men revealed that the gene targets of epigenetic silencing were 5-fold more variable case-to-case than site-to-site ($p < 0.0001$), providing statistical evidence that abnormal DNA methylation patterns may arise before prostate cancer cell growth and expansion at metastatic sites (4).

Somatic DNA hypomethylation has also been described in prostate cancer cell DNA, but has not been studied in as great a detail thus far as somatic hypermethylation. An early analysis of total 5-mC base levels suggested that DNA hypomethylation might be rare in primary prostate cancers, but more common in prostate cancer metastases (77). A subsequent study revealed decreased CpG dinucleotide methylation at *LINE-1* sequences in 53% of all the prostate cancer cases analyzed, with *LINE-1* hypomethylation changes present in 67% of cases with lymph node metastases, but only 8% of cases without lymph node metastases (78). When DNA hypomethylation has been assessed along with CpG island hypermethylation changes at *GSTP1*, *RAR β 2*, *RASSF1a*, and *APC* in prostate cancers, the hypermethylation changes seemed likely to have preceded the hypomethylation

changes, which were generally detected in cancers of higher stage and histologic grade (79). In addition, a provocative correlation between DNA hypomethylation and losses or gains of sequences on chromosome 8 has been described in prostate cancers, consistent with a possible contribution of decreased methylation to genetic instability (45,80).

7. SENSITIVE DETECTION OF HYPERMETHYLATED CPG ISLANDS AS PROSTATE CANCER BIOMARKERS

The use of serum assays for prostate-specific antigen (PSA) as a prostate cancer screening tool has dramatically changed the natural history of prostate cancer, and may be responsible, in part for the recent decline in prostate cancer mortality. As a result of PSA screening, prostate cancer, which once first became evident when complicated by symptomatic metastases, now more typically presents as a localized tumor suitable for treatment by radical prostatectomy or with radiation therapy (81). Nonetheless, PSA screening is far from perfect: in the Prostate Cancer Prevention Trial (PCPT), 24.4% of men on the placebo treatment arm who entered the study with “normal” serum PSA values and underwent prostate biopsies at the end of the trial were found to have prostate cancer (82,82). The current approach to prostate biopsy for prostate cancer detection and diagnosis also leaves a lot to be desired: the typical ultrasound-guided biopsy strategy features random sampling of ~0.3% of prostate tissue, rather than biopsies targeted at some sort of radiographic image abnormality like for other cancers. Also, controversies remains concerning the optimal number of tissue cores that should be obtained during a prostate biopsy procedure, and about which men should be subjected to repeat biopsy procedures if cancer is not detected (84,85). Furthermore, because prostate cancers may be present in more than half of all men over age 50 years, yet threaten morbidity and/or mortality in only 5% or less of men, the wisdom of prostate cancer screening and early detection has been questioned (86). To confront these challenges, new molecular biomarkers have been sought that could be useful for prostate cancer prostate cancer screening, for improving the detection and diagnosis of localized prostate cancer, and for directing treatment choices for men with prostate cancer.

Somatic epigenetic alterations offer a great source of potential molecular biomarkers for prostate cancer and for other human cancers for several reasons: (i) somatic hypermethylation of CpG island sequences have been consistently associated with virtually all human cancers, including prostate cancers, (ii) CpG island methylation can be readily detected in genomic DNA using very sensitive and specific polymerase chain reaction (PCR) strategies, (iii) genomic DNA may be superior to RNA, protein, and other macromolecules in terms of stability for biospecimen collection and handling, and (iv) CpG island methylation changes appear to be more consistently present in different cancer cases, particularly in different prostate cancer cases, than somatic genetic changes, such as mutations, deletions, and translocations,

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permitting one or a few assays to be used as a test (2,4,66,87,88).

There are now three major strategies for the detection of CpG dinucleotide methylation changes in genomic DNA from cancer cells. The first approach features the use of restriction endonucleases that cut recognition sites differently if the sites contain ^{5-mc}CpG. Such enzymes have been used along with Southern blot analysis and with PCR to discriminate DNA methylation changes at particular genome sites (89,90). Assays using ^{5-mc}CpG-sensitive restriction enzymes and PCR (RE-PCR) have proven spectacularly sensitive, capable of detecting single hypermethylated CpG island sequences, but appear prone to false-positive results, arising from incomplete cutting of unmethylated sequences and insufficient suppression of PCR amplification of unmethylated CpG island alleles (90). The second strategy uses sodium bisulfite modification to facilitate the selective deamination of C, but not of ^{5-mc}C, to U, creating a DNA sequence difference at C versus ^{5-mc}C after PCR amplification. This approach has been used for mapping and sequencing of ^{5-mc}C at specific genome sites, and serves as the basis for a PCR assay in which primers specific for bisulfite/deamination converted sequences containing ^{5-mc}C versus C are used to detect hypermethylated CpG islands (91,92). The bisulfite modification and PCR (MS-PCR) assays, though often quite specific, can be less sensitive than RE-PCR assays because the bisulfite modification procedure can damage target DNA sequences (5). A third approach involves selective capture of ^{5-mc}C-containing sequences with ^{5-mc}C-binding proteins or anti-^{5-mc}C antibodies (93,94,95,96,97). New assays featuring capture of ^{5-mc}C-containing DNA appear sensitive, specific, easily adapted to high-throughput analysis platforms, and able to be used in association with RE-PCR and/or MS-PCR methods (eg. COMPARE-MS) (90).

Epigenetic silencing of *GSTP1*, encoding the π -class glutathione *S*-transferase (GST), is nearly ubiquitously associated with prostate cancer (64,65,68). The CpG island encompassing the *GSTP1* transcriptional promoter is devoid of ^{5-mc}C in normal cells of the prostate and other tissues, but in almost all prostate cancers that have been carefully studied, the *GSTP1* CpG island is densely methylated and the gene is transcriptionally silent (68,70,98). Several different detection strategies have been used to detect *GSTP1* CpG island hypermethylation: in a recent review of some 24 published studies with 1071 prostate cancer cases, *GSTP1* CpG island hypermethylation was found in prostate cancer DNA from more than 81% of the cases analyzed (5). The sensitivity of assays for *GSTP1* CpG hypermethylation varied substantially depending on the assay strategy used and the specific region of the *GSTP1* CpG island targeted (5). Using COMPARE-MS, one of the new ^{5-mc}C-containing DNA capture assays, *GSTP1* CpG island hypermethylation exhibited 99.2% sensitivity and 100% specificity for DNA from prostate cancer versus normal prostate tissue (90). For this reason, several different *GSTP1* CpG island hypermethylation assays are under clinical development as tools for prostate cancer detection and diagnosis. Using *GSTP1* CpG island

hypermethylation as a molecular biomarker of prostate cancer, prostate cancer cells, or cell-free prostate cancer DNA, has been detected in prostate tissue biopsies, in prostate secretions or in the urine, and in the circulation (99,100,101,102).

Along with *GSTP1* CpG island hypermethylation, somatic CpG island hypermethylation changes at other loci, including *APC*, *RASSF1a*, *PTGS2*, and *MDR1*, may also help discriminate prostate cancer from non-cancerous tissue with high sensitivity (97.3%–100%) and specificity (92%–100%) (4,5). Undoubtedly, new epigenetically-silenced genes will be added to this list in the future. As for genes carrying somatic DNA methylation changes as prognostic biomarkers for prostate cancers, hypermethylation of CpG island sequences at *EDNRB*, *RARB*, *RASSF1a*, *ERβ*, and *TIG1* have been correlated with known prognostic factors for primary prostate cancer such as tumor stage and/or grade (4,103,105,106). In one study, differences in *PTGS2* CpG island hypermethylation in the primary prostate cancer lesion independently predicted prostate cancer recurrence after radical prostatectomy, even when tumor grade and stage were considered (4). These genes, and others yet to be discovered, may be targets for epigenetic silencing which contribute to a more malignant phenotype of prostate cancer. Detection of CpG island hypermethylation at such a gene locus might then not only provide a biomarker of high-risk prostate cancer, but also provide insight into molecular pathways of malignant prostate cancer progression. For example, *EDNRB* encodes the endothelin-B receptor, a clearance receptor for endothelin-1, produced at high levels by metastatic prostate cancer as part of autocrine and paracrine signaling loops (107,108,109). Loss of this clearance receptor permits unfettered activation of the endothelin-A receptor, a likely participant in the pathogenesis of osteoblastic bony prostate cancer metastases (110,111,112). Atrasentan, an endothelin-A receptor antagonist has shown promise for prostate cancer treatment in randomized clinical trials (113,114). Perhaps, epigenetic silencing of *EDNRB* might define prostate cancer cases for which endothelin-1 signaling loops contribute to disease progression, and help identify cases for which atrasentan might provide a clinical benefit. Otherwise, *GSTP1* CpG island hypermethylation changes are so common in prostate cancer cells, and so rare elsewhere, that detection of DNA with such changes in blood, lymph nodes, bone marrow, and other sites remote from the prostate likely provides evidence that prostate cancer cells may be at such sites. Thus, using *GSTP1* CpG island methylation assays for “molecular staging”, the detection of DNA with *GSTP1* CpG island hypermethylation in the serum of men with localized prostate cancer was associated with an increased risk of prostate cancer recurrence after radical prostatectomy (102).

So far, the use of assays for somatic alterations in DNA methylation in prostate cancer cells as tools for prostate cancer detection, diagnosis, and prognosis has been largely restricted to CpG island hypermethylation changes, with little attention to other epigenetic alterations, including genomic hypomethylation and loss of fidelity for

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genetic imprinting. As more robust assays for these alterations become available, the correlations between these DNA changes and prostate cancer behavior can be tested, perhaps providing new molecular biomarkers for the disease.

8. Epigenetic Gene Silencing as a Therapeutic Target for Prostate Cancer Prevention and Treatment

Epigenetic genome alterations presents some of the most attractive rational targets for all of human cancer, including prostate cancer, because although somatic changes in DNA methylation may lead to gene dysregulation that can be passed through mitosis, unlike somatic mutations and deletions, the base sequence of DNA is not corrupted. Thus far, attempts at therapeutic intervention have focused on epigenetic gene silencing via two major approaches: one targeting over-methylation of CpG island sequences at the promoters of inactivated genes, the other antagonizing the assembly of repressive chromatin structure that prevents the transcription of critical anti-cancer genes. To reduce abnormal CpG island hypermethylation, several inhibitors of DNMTs have been discovered, rediscovered, and developed, including the nucleoside analogs 5-aza-cytidine (Vidaza®), which was approved by the U.S. Food and Drug Administration (FDA) for the treatment of myelodysplasia, 5-aza-deoxycytidine (decitabine or Dacogen®), and zebularine, as well as the non-nucleosides procainamide and hydralazine (115,116,117,118,119,120). For prostate cancer, the results of a small phase II clinical trial (n = 14 men) of decitabine treatment for androgen-independent, progressive, metastatic prostate cancer has been reported weeks (121). In the trial, decitabine was administered at a dose of 75 mg/m² intravenously every 8 hours for three doses, repeated every 5 to 8 weeks, dose and schedule more optimized to tolerability than to the pharmacodynamic goal of a reduction in CpG dinucleotide methylation at the loci of critical cancer genes. Nonetheless, 2 of the 12 men who could be assessed for a treatment response exhibited stable disease, progressing after 10 weeks or so (121). Nucleoside analogs act to inhibit DNMTs when incorporated into genomic DNA, trapping the enzyme into a covalent reaction intermediate that mimics a protein-DNA adduct, promoting the proteolytic destruction of the enzyme as well as activating genome damage responses and cell death pathways (122). This general mechanism of enzyme inhibition presents several safety concerns which will limit the use of nucleoside DNMT inhibitors for applications such as prostate cancer prevention: in addition to myelotoxicity associated with the toxic effects of the inhibitors to dividing bone marrow cells, the incorporation of abnormal nucleosides into genomic DNA might also lead to mutations and future cancer development¹²³.

Non-nucleoside DNMT inhibitors might be free of such safety worries. For example, procainamide, a drug approved by the FDA for the treatment of cardiac arrhythmias, and hydralazine, a drug approved for the treatment of hypertension, both inhibit DNMTs but do not generally cause myelotoxicity or mutations (124,125,126). Furthermore, mechanistic studies of procainamide as a

DNMT inhibitor have revealed some interesting features of the drug that may provide an even more substantial safety margin. First, procainamide appears to be a selective inhibitor of DNMT1 at concentrations that can be achieved clinically, with little or no activity toward DNMT3a or DNMT3b (126). For this reason, the drug may have somewhat limited activity in the treatment of some established cancers, as among different cancer cell lines, genetic knockout or knockdown of DNMT1 exhibits a variable propensity to reactivate epigenetically silenced genes, likely confounded by the ability of DNMT3a or DNMT3b to substitute for DNMT1 in certain situations (127). Of note in this regard, procainamide was able to reactivate silenced *GSTP1* expression in LNCaP prostate cancer cells *in vitro* and *in vivo*, with a trend toward greater antitumor activity than decitabine (118). Second, procainamide only interferes with the activity of DNMT1 on hemimethylated DNA templates (its maintenance DNA methylation function) and does not inhibit DNMT1 activity on unmethylated DNA templates (*de novo* methylation function). This is of interest as mice carrying one disrupted *Dnmt1* allele and one hypomorphic *Dnmt1* allele, resulting in 10% of normal DNMT activity, have been reported to exhibit genomic instability and to develop T-cell lymphomas, hinting that inhibition of DNMT1 activity might promote the appearance of certain cancers (*eg.* lymphomas) even while attenuating the appearance of others (*eg.* epithelial tumors; see 44,45). However, these mice have both reduced maintenance and reduced *de novo* DNMT1 activities. Whether preservation of *de novo* DNMT1 function might limit genetic instability has not been tested, but is nonetheless mechanistically plausible. Long-term use of procainamide has not been associated with genetic instability or with an increased lymphoma risk, although prolonged procainamide treatment has been correlated with drug-induced lupus, more commonly arising in women than in men. In animal models, both nucleoside DNMT inhibitors and procainamide can cause autoimmunity (125,128). Could procainamide be safe enough to consider for prevention of prostate cancer in adult men? More preclinical and clinical/epidemiological data are needed to answer this question.

The other general therapeutic approach to epigenetic gene silencing in cancer features the targeting of enzymes and other proteins that contribute to the construction of a repressive chromatin complex at the regulatory regions of key cancer genes. Such candidate drug targets include histone deacetylases (HDACs), histone methyltransferases (HMTs), and MBDs. The greatest progress thus far has been in the discovery and development HDAC inhibitors, including sodium phenylbutyrate, valproic acid, suberoylanilide hydroxamic acid (SAHA), pyroxamide, *N*-acetyl dinaline (CI-994), LAQ824, LBH-589, MS-275, depsipeptide (FR901228), and many others (129,130,131,132). Several of the HDAC inhibitors have exhibited promising pre-clinical activity in cancer models, including prostate cancer models (133,134,135,136,138, 139,140,141). The early clinical experience with these agents appears quite promising: though side effects, such as nausea, vomiting, diarrhea, fatigue, and edema have been reported, severe adverse

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events appear rare (129,130,132). As a result, combinations of HDAC inhibitors and cytotoxic chemotherapy drugs, radiation therapy, and other chromatin-targeted agents will likely be pursued in preclinical studies and in clinical trials. As an example, combinations of DNMT inhibitors and HDAC inhibitors appear to more effectively trigger silenced gene reactivation in cancer cells (58,142). The suitability of HDAC inhibitors for prostate cancer treatment is under active clinical assessment. As for other chromatin targets, such as HMTs and MBDs, no candidate drugs are yet available, though for MBD2, genetic studies provide provocative target credentialing evidence: MBD2 binds the *GSTP1* promoter when it is methylated in cancer cells and knockdown of MBD2 reactivates *GSTP1* in spite of the DNA methylation change (14,16,68). In addition, *Mbd2*^{-/-} mice appear fairly normal, and *Apc*^{Min/+}*Mbd2*^{-/-} mice develop fewer intestinal adenomas, and survive longer, than do *Apc*^{Min/+}*Mbd2*^{+/-} or *Apc*^{Min/+}*Mbd2*^{+/+} mice (15,143).

9. SUMMARY

Epigenetic alterations are the most common somatic genome changes in prostate cancer cells, associated with defects in gene function that contribute to carcinogenesis and to maintenance of a malignant phenotype. Most of the known epigenetic changes are manifest as abnormal DNA methylation patterns at the transcriptional regulatory regions of key genes. The DNA methylation changes, detected using sensitive PCR methods, are poised to gain early acceptance as molecular biomarkers for prostate cancer detection, diagnosis, and prognosis. New treatment approaches are under development to reverse or antagonize epigenetic gene silencing in prostate cancer and in many other cancers. The first such agent to earn FDA approval is the DNMT inhibitor 5-aza-cytidine (Vidaza®). In the future, epigenetic therapies may be useful both for cancer treatment and for the prevention of the disease.

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Send correspondence to: William G. Nelson, M.D., Ph.D., Room 151 Bunting-Blaustein Cancer Research Building, 1650 Orleans Street, Baltimore, MD 21231-1000; Phone: 410-614-1661; Fax: 410-502-9817; E-mail: bnelson@jhmi.edu

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