Prostate cancer epigenetics and its clinical implications

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Normal cells have a level of epigenetic programming that is superimposed on the genetic code to establish and maintain their cell identity and phenotypes. This epigenetic programming can be thought as the architecture, a sort of cityscape, that is built upon the underlying genetic landscape. The epigenetic programming is encoded by a complex set of chemical marks on DNA, on histone proteins in nucleosomes, and by numerous context-specific DNA, RNA, protein interactions that all regulate the structure, organization, and function of the genome in a given cell. It is becoming increasingly evident that abnormalities in both the genetic landscape and epigenetic cityscape can cooperate to drive carcinogenesis and disease progression. Large-scale cancer genome sequencing studies have revealed that mutations in genes encoding the enzymatic machinery for shaping the epigenetic cityscape are among the most common mutations observed in human cancers, including prostate cancer. Interestingly, although the constellation of genetic mutations in a given cancer can be quite heterogeneous from person to person, there are numerous epigenetic alterations that appear to be highly recurrent, and nearly universal in a given cancer type, including in prostate cancer. The highly recurrent nature of these alterations can be exploited for development of biomarkers for cancer detection and risk stratification and as targets for therapeutic intervention. Here, we explore the basic principles of epigenetic processes in normal cells and prostate cancer cells and discuss the potential clinical implications with regards to prostate cancer biomarker development and therapy.

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INTRODUCTION TO EPIGENETICS: BEYOND GENETICS

The field of epigenetics, once a very specialized area of study only undertaken by specialists, has now come to pervade nearly all fields of study in biomedical research. This is in large part because of the increasing recognition of the importance of epigenetics to health and disease. Along with this increased awareness, however, there is often some confusion as to what exactly epigenetics refers to. We will begin this chapter with a brief overview of the basic concepts and principles of epigenetics, and then delve into recent insights on prostate cancer epigenetics and their potential for translation to biomarkers and therapeutic strategies.

Epigenetic programming helps establish, alter, and maintain cell identity and cell function

Epigenetics can be defined as the study of processes that mediate potentially heritable (here, heritable refers to passing down information from parent cells to daughter cells rather than from parents to children) changes in cellular phenotypes without a change in the genetic sequence. To better understand this, let’s consider two scenarios where there are clearly major changes in cellular phenotypes but without any systematic changes in genetic sequence. First, for a given individual, throughout development, from the single cell stage, through all stages of fetal development, neonatal life, childhood, adolescence, adulthood, and aging, it is apparent that cells have ever-changing cellular phenotypes. Second, at any given stage in life, each organ and cell type in the body can have very different functions and gene expression programs, and each time those cells divide or regenerate, they have to “remember” or maintain those gene expression programs. These differences in cellular phenotypes over time and across organ/cell types are established and maintained despite the fact that the genetic sequence is largely constant. Therefore, there must be some level of programming beyond the genetic sequence that mediates the establishment and maintenance of cell identity over time and across cell types – we call the study of that level of programming epigenetics (beyond genetics, from the greek/latin epi meaning beyond or above).

The major epigenetic marks and their mediators constituting a cell intrinsic epigenetic code in normal and cancer cells

The epigenetic code is established, maintained, and dynamically regulated by a set of epigenetic machinery proteins and other macromolecules (e.g., noncoding RNAs). Broadly speaking, we can classify this machinery that mediates the epigenetic programming as “writers,” “erasers,” “readers,” and “preservers” (Table 1). As a prerequisite for understanding how these functions and the epigenetic marks are altered in prostate cancer, we will briefly discuss the various marks and the epigenetic machinery here. A more exhaustive dissection of these processes has been described in numerous previous reviews.1-3

By regulating this machinery, cells can establish and maintain their epigenetic programming or can dynamically alter them. There is thus a fundamental paradox in the epigenetic code: cells can choose to stably pass down marks and programming or they can dynamically alter them by reversing the marks and establishing new marks. The precise
details of these mechanisms have not been worked out but have been best understood so far for DNA methylation, which is where we can begin our dissection.

**DNA methylation**

Methylation of the 5-position of the cytosine base is an important epigenetic mark in human DNA (and highly conserved through vertebrates; plants and other eukaryotes also utilize such cytosine methylation-based epigenetic regulation). In normal adult cells, the majority of 5-methylcytosine marks occurs in the context of cytosine-phospho-guanine (CpG) dinucleotides, with >80% of such CpG's typically being methylated. While cytosines in other sequence contexts can occur relatively frequently in embryonic stem cells, such non-CpG methylation usually accounts for <1% of overall cytosine methylation in the majority of adult cell types studied but may have some functional significance despite its low abundance. CpG methylation in normal cells is typically excluded from CpG dense regions in the genome called CpG islands that are often found around transcriptional start sites of genes and other regulatory regions. In cancer cells, these CpG islands, and regions with a lower CpG density around these CpG islands, called “CpG island shores,” can be abnormally hypermethylated in cancer cells compared to normal cell counterparts. This CpG island hypermethylation is often associated with repression of the nearby gene and has become recognized as a major mechanism of epigenetic gene repression that is particularly associated with silencing of cancer-protective genes as well as genes involved in development and differentiation in cancer cells. This DNA hypermethylation-mediated gene repression can be quite stable, inactivating genes in a manner analogous to genetic loss of function mutations and deletions. Paradoxically, in parallel with development of DNA hypermethylation-mediated epigenetic repression at CpG islands and shores around gene regulatory regions, cancer cells can also show progressive hypomethylation of many CpG dinucleotides. In large stretches of genomic DNA often spanning many hundreds of kilobases to megabases, where normal cells harbor a high degree of CpG methylation, cancer cells often exhibit diminished methylation. While this type of hypomethylation does not seem to lead to activation of genes nearby with any consistency, it is thought that such genomic blocks of hypomethylation can be associated with more open chromatin in cancer cells, leading to genomic instability. In addition, this hypomethylation has been associated with activation of endogenous retroviral elements, retrotransposons, and other repetitive sequences, further contributing to genomic instability.

The “writers” of the DNA methylation code are the DNA methyltransferase enzymes, and in human cells, they are encoded by three genes, *DNMT1*, *DNMT3A*, and *DNMT3B*. A related gene, *DNMT3L*, does not possess catalytic activity on its own but can complex with DNMT3A and DNMT3B to assist with their catalytic activity. DNMT1 is the major DNA methyltransferase required for maintaining DNA methylation marks across replication. It tracks with the replication fork and is particularly adept at methylating CpG dinucleotides in the daughter strands when the palindromic CpG dinucleotide in the parental strand was methylated. There is also a great degree of cooperativity between DNMT1 and DNMT3 isoforms in carrying out this maintenance methylation, particularly at repetitive sequences. DNMT3A and DNMT3B have often been referred to as “de novo” DNA methyltransferases in part because they can methylate completely unmethylated DNA to an equal catalytic efficiency as they can hemimethylated DNA. They have been shown to be important for de novo methylation in various biological contexts including embryonic stem cells and other settings of development and differentiation. DNA methylation can be “passively” lost across DNA replication if the machinery fails to “copy” the CpG methylation from the parent strand onto the daughter strand.

DNA methylation can also be actively “erased,” presumably independent of DNA replication. Such active reversal of DNA methylation has been suggested to play an important role in mediating plasticity and dynamic epigenetic regulation, particularly in certain cell types such as neurons and embryonic stem cells. Recent work has implicated oxidative demethylation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine by ten-eleven translocation (TET) family proteins, encoded by three distinct genes such as *TET1*, *TET2*, and *TET3*. Although the precise mechanism of active demethylation by such oxidation has not been fully worked out, it appears that the oxidized forms are either directly excised by DNA glycosylases such as TDG and replaced with unmethylated cytosine by base excision repair (BER) machinery or are first deaminated prior to excision and BER. Haﬀner and colleagues were the ﬁrst to show robustly that when compared to normal differentiated/efﬁctor cells in many tissue types, the abundance of 5-hydroxymethylcytosine, which is the major oxidized form of 5-methylcytosine, is profoundly reduced in human cancers, including prostate cancer, and stem/progenitor cell compartments. This ﬁnding has now been reproduced by numerous labs for a multitude of cancer types. We speculate that this loss of 5-hydroxymethylcytosine in cancer cells might indicate a reduced plasticity of DNA methylation alterations in human cancer. Among the “readers” of DNA methylation marks are specialized methyl-binding domain proteins capable of selectively binding methylated DNA and recruiting corepressors complexes such as Mi2-NURD to those sites to transduce the DNA methylation signal and produce repressive chromatin. In human cells, there are three well-characterized methyl-binding domain proteins, encoded by *MBD1*, *MBD2*, *MBD3*, and *MECP2*. These, MB1, MB2, and MECP2 show high afﬁnity for methylated CpG dinucleotides in double-stranded DNA. Although MB3 is highly homologous to MB2, it does not display afﬁnity for methylated DNA but is thought to be an integral member of corepressor complexes involved

**Table 1: Overview of the epigenetic machinery: The mediators of key epigenetic marks. These mediators are being explored as targets for cancer epigenetic therapy**

| Writers | Erasers | Readers | Preservers |
|---------|---------|---------|------------|
| DNA methylation | DNA methyltransferases (e.g., DNMT1, DNMT3A, DNMT3B) | TET enzymes and BER machinery (e.g., TET1, TET2, TET3, TDG) | MBD, zinc ﬁnger proteins (e.g., MBD1, MBD2, MECP2, ZBTB33) | UHRF1, DNMT1 |
| Histone methylation | Histone methyltransferases (e.g., MLL1, EZH2, DOT1L) | Histone demethylases (e.g., KDM1A, KDM6A, KDM4B, KDM4C, KDM2A) | Chromodomain, PHD ﬁnger proteins | UHRF1, unknown |
| Histone acetylation | Histone acetyltransferases (e.g., p300/CBP) | Histone deacetylases (e.g., HDAC1, HDAC2, HDAC3, HDAC4, HDAC6) | BET bromodomain proteins (e.g., BRD4, BRD2, BRD3) | Unknown |
in repressing chromatin. A recent study has suggested that MBD3 may bind hydroxymethylated DNA,\textsuperscript{33} but this has been controversial, and more recent work has implicated MECP2 as the major MBD protein capable of binding hydroxymethylcytosine marks,\textsuperscript{44} raising the possibility that hydroxymethylcytosine may not merely be an intermediate oxidized form leading to demethylation but may have its own signaling functions distinct from 5-methylcytosine. Finally, MBD4 has a glycosylase domain and is thought to be involved in base excision repair of T:G mismatches produced by deamination of 5-methylcytosine.\textsuperscript{35}

Of the different epigenetic marks, our understanding of the mechanisms by which DNA methylation alterations are heritably maintained, via the action of "preservers," across DNA replication and cell division is most mature. As discussed above, DNMT1 can track with the replication fork and has strong catalytic activity for methylation of the daughter strand when the parent strand is methylated at a CpG dinucleotide. In addition, the UHRF1 protein, which is expressed during S phase, can bind hemimethylated DNA at the replication fork and can recruit DNA methyltransferases to those sites to enforce maintenance of methylation during replication.\textsuperscript{36-40} With these mechanisms combined, there appears to be a robust potential for maintaining DNA methylation in human cells. Interestingly, for other epigenetic marks, such as histone modifications, the best-known mechanisms for maintenance across replication involve signaling and reinforcement by DNA methylation. For example, UHRF1 can also recruit the G9A histone methyltransferase to methylate H3K9 at sites of DNA methylation across replication.\textsuperscript{41,42} Such mechanisms closely link the various epigenetic marks to mediate a unified and cooperative epigenetic code.

**Histone modifications and DNA, RNA, protein interactions**

Another important set of epigenetic marks are those found on DNA-binding proteins. The best understood of these are the marks on histone proteins in nucleosomes. Each of the major nucleosomal histone subunits, H2A, H2B, H3, and H4, have long polypeptide tails that protrude from the tight barrel-like structure of DNA wrapped around the histone octamer. These protruding tails are especially prone to numerous posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, and many others. The precise location of the modification as well as the type of modification appears to establish a "histone code" that instructs cellular machinery to maintain the associated chromatin in repressive, active, or poised states.\textsuperscript{43,44} However, given the numerous modifications and the fact that these modifications can occur in innumerable combinations, this "histone code" appears to be unimaginably more complex than the simple genetic code. Compounding this enormous complexity, a significant portion of the products of protein-coding as well as noncoding RNA genes bind to and regulate genome structure and function. Such interactions include those with insulators such as CTCF,\textsuperscript{44} long-noncoding RNAs,\textsuperscript{45} linker histones, transcription factors, coactivators and corepressors, and many others.

The most studied marks comprising a putative histone code are histone methylation and histone acetylation. Histone tail acetylation is typically associated with open or active chromatin. Histone methylation of the basic amino acids such as Lysine and Arginine on histone tails can be associated with either open or closed chromatin, depending on the location and number of methylation events at that location.\textsuperscript{44,45} For example, methylation of H3K27 (the lysine present at the 27\textsuperscript{th} position of H3), denoted H3K27me, is usually associated with repressed chromatin. Methylation of H3K9 is also typically associated with repressed chromatin. However, methylation of H3K4 is typically associated with open or active chromatin and is found near actively transcribed genes. Recently, it has also been shown that both active and repressed chomatin methylation marks can be found at the same locus, a type of "bivalent" signal that keeps the chromatin in a "poised" state in embryonic stem cells and other tissue stem cells.\textsuperscript{46} As these cell differentiate, the poised state transitions to a more committed state carrying either the open or closed chromatin methylation mark alone.

Each histone modification is regulated by a distinct set of "writers," "erasers," and "readers." For histone methylation and acetylation, the writers are histone methyltransferases (HMT) and acetyltransferases (HAT), respectively, and the erasers are histone demethylases (HDM) and histone deacetylases (HDAC), respectively. The readers of histone modifications, responsible for selectively binding those modifications and transducing the signal by recruiting appropriate effector activation and repression complexes, are only now beginning to be understood. The BET bromodomain proteins are the best studied of these and are involved in transducing the signal of histone acetylation to promote gene activation and open chromatin.\textsuperscript{47,48}

With this daunting complexity, a comprehensive understanding of the histone code will require the development of novel technologies to study the combinations of marks present at each locus in the genome across multiple different functional states. With the burgeoning development of genomics and proteomics technologies, coupled with novel computational approaches in systems and network biology, the field will need to develop a conceptual framework for understanding the histone code.

**EPIGENETIC ALTERATIONS ARE UNIVERSAL IN PROSTATE CANCER**

Even if the precise mechanisms are not fully known, it is becoming clear that epigenetic marks and processes are universally altered in human cancers, including prostate cancer. These alterations are manifest in all of the epigenetic marks studied to date and can be widespread across the cancer genome, altering thousands of loci. This reinforces the central importance of epigenetic mechanisms in allowing cancer cells to evade the rules that typically govern normal cells, and establish a new cell identity and function that enables self renewal, survival, and invasion. We will next examine the known epigenetic alterations in human prostate cancer.

**DNA methylation alterations in prostate cancer**

Of the epigenetic marks, our understanding of DNA methylation alterations in prostate cancer is most mature. Early in prostate carcinogenesis, during the transition from benign prostate epithelium to inflammatory lesions called proliferative inflammatory atrophy and premalignant prostatic intraepithelial neoplasia lesions, we can first observe evidence of DNA hypermethylation of promoter regions of key tumor suppressor and cancer caretaker genes such as GSTP1, RASSF1A, and APC.\textsuperscript{49,50} As these lesions transition to invasive adenocarcinoma, genome-wide and candidate gene studies of DNA methylation have indicated that there appears to be a DNA hypermethylation catastrophe, involving hundreds to thousands of CpG-dense regions (e.g., CpG islands and CpG island shores) in the prostate cancer genome.\textsuperscript{48,49} Such alterations are among the most numerous and recurrent somatic genome alterations known in prostate cancer, even more so than the recently characterized recurrent rearrangements involving ETS transcription factors in prostate cancer.\textsuperscript{52} These hypermethylated regions are highly enriched for promoter elements and can be associated with silencing of the associated gene.\textsuperscript{51} Such genes are in turn enriched for development and differentiation.
pathways, suggesting that DNA hypermethylation may be involved in suppressing differentiation programs.\(^5\) In addition to being enriched for promoter regions near transcriptional start sites, the genome-wide DNA methylation alterations are also highly enriched for proximity to transcriptional termination sequences and intron-exon junctions and exon sequences.\(^5\) Whether such DNA hypermethylation causally affects gene expression patterns, for example, by regulating anti-sense transcription, alternative polyadenylation site usage, or splicing of alternative transcriptional isoforms, has been raised as a possibility;\(^5\) but remains largely speculative. In addition to these gene-associated DNA methylation alterations, genome-wide studies have also identified numerous intergenic DNA methylation alterations. While the functional significance of these alterations is difficult to understand, it is interesting to note that such intergenic hypermethylated regions in prostate cancer are highly enriched for conserved sequences and conserved transcription factor binding sites, suggesting that they may influence gene regulation at a distance. For a subset of such regions, it is likely that they directly suppress transcription of previously unappreciated noncoding RNAs.\(^5\) As our understanding of the functional elements in the genome improves with large-scale efforts such as the ENCODE project\(^5\) and the Epigenomics Roadmap project,\(^5\) the field will be better able to understand the mechanistic significance of such alterations.

While the majority of DNA hypermethylation alterations appears to occur during the early catastrophe described above, it is also apparent that multiple subsequent waves of DNA hypermethylation likely accompany, and perhaps participate, in driving disease progression and therapy resistance.\(^4\) Numerous studies have identified DNA methylation alterations associated with high-stage and/or stage and have also identified DNA hypermethylation changes that are associated with disease recurrence after primary therapy.\(^2,4,5\) More recently, DNA hypermethylation alterations associated with neuroendocrine prostate cancer and lethal metastatic prostate cancer have been identified.\(^2,5\) Such alterations may be involved in emergence of resistance to first- and second-generation anti-androgen therapy and chemotherapy.

As with many other cancer types, prostate cancers can also harbor significant hypomethylation, i.e., undermethylation at genomic regions that are universally methylated in normal prostate tissues.\(^4,11,35\) However, unlike many other cancer types in which DNA hypomethylation appears to be an early event accompanying carcinogenesis, for prostate cancer, such hypomethylation appears to occur late in disease, most prominent in metastases.\(^4,11,35\) These hypomethylation alterations can affect large blocks of the genome, spanning hundreds of kilobases to megabases in size, punctuated by regions of hypermethylation at small regions of just a few hundred base pairs.\(^2\) The hypomethylation alterations do not appear to influence gene expression in cis in any consistent direction, except for a handful of cancer testis antigens (so-called because of their typical expression pattern in normal testis and in multiple cancer tissues), which are variably hypomethylated in their promoter regions associated with overexpression.\(^4,11\) DNA hypomethylation can occur at repetitive elements, including retropseudonpon and retroviral elements.\(^11,28\) Despite their lack of association with cis gene regulation, DNA hypomethylation has been proposed to lead to genetic instability by promoting formation of open chromatin that can be prone to damage by genotoxic stress and can be more recombinogenic, particularly when occurring at repetitive regions in the genome. The hypomethylation of L1 and Alu elements has also been suggested to activate these elements, potentially leading to retrotransposition mediated genetic instability.\(^11\)

To better understand the complex spectrum of DNA methylation alterations in prostate cancer, our group recently created the first genome-scale DNA methylation “cityscapes” of lethal metastatic prostate cancer.\(^1\) In this study, the genome-wide DNA methylation patterns of multiple anatomically distinct metastatic deposits from a rapid autopsy study of men with lethal metastatic prostate cancer were determined and compared to DNA methylation patterns of normal prostate tissues. Because each metastatic deposit represents a distinct clonal expansion, the degree to which DNA methylation alterations were maintained across metastatic dissemination could be assessed. Interestingly, DNA methylation alterations overall were highly maintained to a similar extent as genetic alterations in copy number.\(^4,5,9\) However, there was a significant fraction of regions that was not as highly maintained across metastatic deposits. Such variably methylated regions were typically regions of hypomethylation, which were widespread across the genome. In this backdrop of widespread and variable hypomethylation, DNA hypermethylation alterations were comparatively more sparse but were highly maintained. The general tendency for loss of methylation in a widespread manner across the genome combined with very high degree of maintenance of hypermethylation suggested that these hypermethylation events were likely selected for and thus enriched for driver events. Consistent with this notion, the highly maintained hypermethylation events were strongly, inversely correlated with the level of expression of adjacent genes. Such regions were also enriched for cancer-associated and development and differentiation pathways, many of which are commonly hypermethylated already in primary prostate cancer. Thus, the hypermethylation alterations in human prostate cancer appear to be highly maintained longitudinally and are likely selected for during disease progression and across metastatic dissemination.

Along with this staunch maintenance of DNA hypermethylation alterations, prostate cancers, like many human cancers, exhibit a profound loss of hydroxymethylation in the genome.\(^1\) We can speculate that this loss of hydroxymethylation in the genome might allow reduced plasticity of DNA methylation and greater potential for maintenance and selection of driver epigenetic alterations.

**Alterations in histone modifications and epigenetic machinery in prostate cancer**

Our understanding of histone modifications in human prostate cancer is still fairly limited, largely due to the lack of robust technologies to enable measurement of the marks genome-wide in human tissues. In prostate cancer and normal cell lines in vitro, using chromatin immunoprecipitation and next generation sequencing or microarrays (ChIP-seq or ChIP-chip), which usually requires large numbers of input cells, it has been suggested that histone modifications undergo an epigenetic switch, in which regions that are marked by the H3K27me polycomb mark in normal cells often exhibit DNA hypermethylation and a switch to H3K9me marks in cancer cells.\(^2\) This switch has been suggested to confer a more stable epigenetic repression of chromatin in cancer cells. This is reminiscent of a more generalized phenomenon across cancers, in which regions that harbor bivalent (H3K4me and H3K27me) marks in embryonic stem cells, and transition to H3K27me3 marks in tissue stem cells, often exhibit DNA hypermethylation and loss of the polycomb mark in cancer cells. This phenomenon has been termed as the DNA hypermethylation module in the polycomb and stem cell signature of cancer cells.\(^1\)

More recently, with refinement of ChIP-seq technologies to allow lower input material,\(^1\) various efforts to measure chromatin marks
and transcription factor binding in prostate cancer are underway.\textsuperscript{53} With the maturation and use of these approaches, it may be possible to observe whether the epigenetic switch and DNA hypermethylation module in the cancer stem cell/polycomb-loss signature will generalize to human prostate cancer tissues.

Efforts to more globally measure the levels of histone modifications have been much more fruitful using immunohistochemistry and immunofluorescence approaches in prostate cancer tissues. Prostate cancers exhibited alterations in the global levels of histone modifications, including histone acetylation (at H3K9, H3K18, and H4K12) and methylation (H3R3me2, H3K4me2), and these alterations fell into distinct patterns within the cancers analyzed.\textsuperscript{54} More notably, prostate cancers exhibit a pronounced and global reduction in the H3K27me3 polycomb marks, and this appears to be reduced even in early stages of the disease and continued in more advanced stages.\textsuperscript{55} Interestingly, this reduction in H3K37me3 marks is highly correlated, even at a cell-by-cell level within prostate cancer tissues, to the loss of hydroxymethylcytosine in prostate cancer tissues (see above), providing additional support that there may be some level of coupling between polycomb marks and DNA methylation in prostate cancer tissues.\textsuperscript{66} This global reduction in the polycomb marks is even more interesting in light of the fact that EZH2, the major catalytic protein responsible for carrying out methylation of the H3K27 residue in the polycomb repressive complex 2 (PRC2), is consistently found upregulated in prostate cancer, particularly in more aggressive and neuroendocrine disease.\textsuperscript{57,58,66} Why this upregulation of EZH2 often accompanies downregulation of the H3K27me mark that it is responsible for “writing” is still largely unknown. A recent report has suggested that in prostate cancer, EZH2 may become phosphorylated and have PRC2-independent function to modulate AR transcriptional programs.\textsuperscript{69}

In addition to upregulation of the epigenetic machinery protein EZH2, it is now evident that several chromatin-modifying proteins can be mutated in prostate cancer. Each of these individual proteins is mutated in only a small fraction of cases, but taken together, chromatin modifying epigenetic machinery proteins are one of the most frequently mutated class of genes in prostate cancer. Such mutations (including point mutations and indels, deletions, and rearrangements), mostly inactivating, have been observed in the machinery regulating DNA methylation (TET2, IDH1), histone modification (MLL2, KDM6A), and chromatin modifiers (ASXL1, ATRX), among several other epigenetic genes with low frequency mutations.\textsuperscript{70–72} The consequences of such mutations are still largely unknown, and future studies focusing on the epigenomic, molecular, functional/mechanistic, and phenotypic consequences of these mutations are critically needed.

**CLINICAL TRANSLATION OF PROSTATE CANCER EPIGENETICS**

Regardless of the functional and mechanistic consequences of the epigenetic alterations in prostate cancer, the high frequency of these alterations in epigenetic marks can provide a rich source of biomarkers. In addition, the mutations and altered expression of epigenetic machinery proteins suggest that the epigenetic machinery may be dysregulated and may present rational targets for prostate cancer therapy.

**Utility of epigenetic alterations as prostate cancer biomarkers**

There are a number of clinical contexts in the management of prostate cancer where there is a critical unmet need for novel biomarkers that may be addressed through translation of our understanding of epigenetic alterations in prostate cancers. These clinical contexts with major unmet clinical needs include (i) screening, (ii) diagnosis, (iii) risk stratification at the time of diagnosis, (iv) disease monitoring during active surveillance, and (v) monitoring disease burden and treatment response, particularly in the setting of androgen deprivation therapy. Several of these unmet clinical needs could potentially be addressed by epigenetic biomarkers (Table 2) as discussed below.

**Prostate cancer screening and diagnosis and monitoring disease burden**

Measurement of serum PSA as a screening tool, although still in widespread use, has been highly controversial.\textsuperscript{73} This is in large part because of its very poor sensitivity, specificity, and predictive values. In addition, there have been major concerns that its widespread use leads to overdiagnosis and overtreatment of otherwise indolent prostate cancer (discussed below). Given the large number of highly sensitive and specific DNA methylation alterations that are cancer specific, and essentially undetectable in benign prostate tissues, DNA methylation alterations, if measurable in cell-free circulating tumor DNA, or in urine, can potentially serve as an important biomarker for prostate cancer screening.\textsuperscript{74} The types of DNA methylation alterations that would be useful in this setting are those that are highly frequent in prostate cancer cells but never found in benign prostate tissues and in the blood and urine of unaffected individuals. Such markers may include CpG island methylation in the regulatory regions of GSTP1, APC, PTGS2, RASSF1A, and RARB, among hundreds of others identified through candidate gene and genome-scale studies of cancer and normal tissues.\textsuperscript{74,75}

These same DNA methylation alterations, if detected in biopsy materials, may also aid in the tissue diagnosis of prostate cancer. A major problem in prostate cancer tissue diagnosis is the use of “blind” biopsies that arbitrarily sample the prostate gland since it is currently not standard of care to use imaging-guided biopsies to specifically sample regions of the prostate that are suspected to have cancer. Given this blind biopsy problem, a negative biopsy result does not necessarily mean an absence of cancer in the prostate – the cancerous region may simply have been missed during biopsy. To address this, there is already a clinically useful test involving the detection of GSTP1, APC, and RASSF1A CpG island methylation in biopsy materials to guide whether a given patient that showed absence of cancer in their biopsies may have molecular evidence for the presence of cancer, and thus be subjected to a rebiopsy.\textsuperscript{75,76} In future, the ability to augment this test with noninvasive detection of DNA methylation alterations in blood and urine may further improve the utility of DNA methylation biomarkers for prostate cancer biopsy.\textsuperscript{76} In addition to these DNA methylation biomarkers, immunohistochemical detection of global alterations in histone modifications and in the level of hydroxymethylcytosine may also have utility in aiding biopsy tissue-based diagnosis of prostate cancer, particularly when morphological features are inconclusive.\textsuperscript{77–79}

**Risk stratification at diagnosis and monitoring during active surveillance**

Current medical practices lead to systematic overdiagnosis and overtreatment of prostate cancer, placing a major unnecessary fiscal burden on the health care system and even causing harm to a significant number of men who receive no benefit from primary therapy. While prostate cancer remains a leading cause of cancer-related deaths in men, claiming nearly 30,000 lives each year, the majority of the nearly 240,000 diagnosed with prostate cancer each year will die with but not from prostate cancer, with many men having disease that will never become life-threatening even without treatment.\textsuperscript{77–81} Recent studies
have estimated that somewhere between 5 and 48 men would need to be detected and potentially treated to prevent one death from prostate cancer. Additional studies have shown that >50% of men receiving radical prostatectomy or radiation therapy for localized prostate cancer develop long-lasting and even lifelong complications such as urinary incontinence and sexual dysfunction. Clearly, there is an urgent need to develop effective noninvasive molecular tests for distinguishing men with aggressive disease that may benefit most from primary therapy versus men with indolent forms of prostate cancer for whom active surveillance paradigms may be more appropriate. Noninvasive molecular risk stratification tests, if deployed longitudinally in active surveillance patients, can also be used to monitor disease progression. With such an advance, physicians and patients could safely avoid overtreatment while ensuring that those patients with aggressive forms of prostate cancer receive potentially curative primary therapy.

The best risk stratification tool in current practice, Gleason grading, when assigned by expert pathologists after examination of prostate tissue resected at radical prostatectomy, is highly effective at prognostication; in a recent study of >2500 men who underwent resection of Gleason 6 or lower prostate cancer, none died of their disease. Therefore, it would seem that men diagnosed with a Gleason 6 or lower cancer may be appropriate for active surveillance. Although there is mounting interest in the US for implementing active surveillance as a viable alternative to radical prostatectomy and radiation therapy, the threat of incorrect classification of cancer aggressiveness during diagnosis and the potential for undetected progression during active surveillance has been a major barrier to widespread adoption. This uncertainty arises because of the use of a "cancer-blind" biopsy prostate; even with the current use of 12-core biopsies, prostate biopsies sample only a very small volume of the prostate and it is very possible that the biopsy may miss aggressive cancers identifiable by pathological examination. Indeed, recent studies have shown that the biopsy Gleason grade and the radical prostatectomy Gleason grade match in only half of all cases. This coupled with inter- and intra-observer variability in assigning Gleason score, particularly in the community setting, causes a great deal of uncertainty in risk stratification. The use of additional biomarkers such as serum PSA and others has added only limited utility. Finally, repetitive biopsy regimens to monitor patients on active surveillance are vulnerable to the same pitfalls as first-time biopsies. In addition to being a somewhat invasive procedure that men often find uncomfortable, biopsies can lead to morbidity such as infection and other complications and can even lead to death in rare circumstances.

This unmet clinical challenge could potentially be addressed by translation of DNA methylation alterations that are associated with aggressive prostate cancer (track with high-grade and metastatic cancer, or with risk of recurrence) into noninvasive biomarkers detectable in circulating cell-free DNA, in circulating tumor cells (CTCs), or in patient urine samples. Several prior reports have nominated DNA methylation alterations associated with prostate Gleason grade, with metastatic disease, and with disease recurrence after primary therapy. However, most of these are smaller studies and have not been sufficiently validated in larger studies. If such alterations are further validated and can be detected by noninvasive tests, they may have tremendous utility to risk stratify patients for aggressive primary therapy, compared to more conservative management with active surveillance. Such noninvasive tests may also be useful during monitoring of patients during active surveillance, reducing the need for repetitive biopsies as the only viable monitoring tool. Application of such tests in biopsy tissues may also be of some utility. In addition, global levels of histone modifications in prostate cancer tissues also appear to be associated with disease aggressiveness and recurrence and may be useful for risk stratification of biopsy tissues.

### Monitoring disease burden and treatment response in the setting of androgen deprivation therapy

There is a critical need for developing molecular biomarkers of treatment response in the settings of androgen deprivation therapy/ radiation therapy for intermediate and high-risk primary prostate cancer and for systemic management of advanced/recurrent prostate cancer where serial serum PSA determinations have proven inadequate. Disease burden and response to therapy in these settings are evaluated by sequential determinations of serum PSA and clinical and radiological parameters. However, these existing strategies are notoriously inadequate, and there are no standard validated response and progression criteria in the setting of systemic management at this time. Currently, serial determination of serum PSA is used as a molecular marker for global monitoring of response to a variety of systemic therapies, but this strategy is fraught with limitations: (i) since PSA is an androgen receptor target gene, androgen deprivation therapy, an initial mainstay of recurrent prostate cancer treatment, will reduce PSA expression simply by down-regulating androgen-receptor signaling, and this effect can not easily be distinguished from a true decrease in prostate cancer cell number and disease burden; (ii) attempts to validate a variety of serum PSA parameters (percent decline, PSA doubling time, changes in PSA slope, etc.) as biomarkers for therapeutic response and/or disease progression during evaluation of new or existing drugs for treatment of advanced prostate cancer have not been successful; and (iii) a number of compounds may modulate PSA expression unrelated to antitumor activity, and this has been a confounding issue in evaluation of new therapeutic agents.
Tumor-specific DNA-based biomarkers, measured from cell-free circulating tumor DNA or in circulating tumor cells, may have a greater potential than serum PSA or other protein or RNA-based biomarkers to stoichiometrically reflect tumor cell number even in the face of intervention-induced molecular signaling alterations that can confound transcript/protein levels independent of changes in tumor burden. Such DNA-based biomarkers have already been in development for measurement of cancer-specific mutations and rearrangements in cell-free circulating tumor DNA. DNA methylation alterations may be particularly well suited in this clinical space since they can be highly cancer-specific, and occur with high frequency in cancer cells. The same types of DNA methylation biomarker panels described above for prostate cancer screening could also have utility for monitoring disease burden and treatment response.

Technologies for detection of DNA methylation alterations as clinically useful biomarkers

Several reports from our group and others have now firmly established the feasibility of detecting prostate cancer-specific DNA methylation biomarkers in bodily fluids such as urine, blood (serum and plasma) and in prostatic secretions. A recent clinical trial has shown that GSTP1, APC, and RARB promoter hypermethylation as detected by the methylation-specific polymerase chain reaction in urine can greatly outperform serum PSA in predicting who will develop a positive prostate cancer biopsy, suggesting that such DNA methylation biomarkers can be detected very early in disease management. In addition, a recent meta-analysis of studies examining numerous reports measuring GSTP1 methylation in plasma and urine using conventional DNA methylation assays such as MSP and methylation-sensitive restriction enzyme-based PCR has demonstrated that this DNA methylation alteration can be found with sensitivities approaching 75% and specificity approaching 95%. Therefore, based on data from our group and from the collective literature, measurement of prostate cancer-specific DNA methylation alterations in blood and urine is highly feasible.

Nonetheless, further improvements and refinements in the technologies used for measurement of DNA methylation alterations could potentially improve biomarker performance for the clinical contexts described above. Currently, the most commonly used technologies rely on sodium bisulfite conversion of cytosine to uracil, a process that spares methylcytosine, thus creating a sequence difference from the DNA methylation alteration. Technologies such as methylation-specific PCR and its derivatives and bisulfite sequencing/pyrosequencing are commonly used tools. However, the process of sodium bisulfite conversion can injure DNA and compromise assay sensitivity. Other bisulfite-free technologies include use of methylation-sensitive restriction enzymes to selectively digest unmethylated but not methylated target sequences followed by qPCR. While this approach can be highly sensitive, failure to digest DNA can lead to false positive identification of DNA methylation, potentially compromising assay specificity. Affinity enrichment of methylated DNA using 5-methylcytosine-specific antibodies or methyl-binding domain (MBD) polypeptides followed by qPCR can also be effective to measure DNA methylation. However, incomplete enrichment and background binding of unmethylated sequences can potentially reduce assay sensitivity and specificity. Interestingly, using a combination of methylated DNA enrichment and methylation-sensitive restriction enzymes (COMPARE-MS) to highly selectively enrich methylated DNA followed by qPCR or other nucleic acid detection technologies can achieve very high assay sensitivity and specificity while avoiding the pitfalls of sodium bisulfite. Whether such an approach for detection of DNA methylation biomarkers in the clinical contexts described above is effective will require rigorous testing.

Potential for targeting epigenetic processes for prostate cancer therapy

As our understanding of epigenetic mechanisms driving prostate carcinogenesis and disease progression advances, the prospect of targeting epigenetic processes for prostate cancer therapy is showing some promise.

DNA methylation alterations are highly frequent, mediate epigenetic repression of key tumor suppressors and caretaker genes, and are stably maintained and appear to be subject to selection during prostate carcinogenesis and disease progression. Existing inhibitors of the DNA methyltransferases, responsible for “writing” these DNA methylation marks, include the nucleoside analog drugs azacitidine, decitabine, and guadecitabine; both azacitidine and decitabine have won FDA approval for treatment of myelodysplastic syndromes. For prostate cancer and other solid organ cancers, these agents have not shown single agent activity. Similarly, HDAC inhibitors have been approved for cutaneous T-cell lymphomas but have not shown significant single agent activity for solid organ cancers. However, in one recent clinical study in nonsmall cell lung cancer, the combination of azacitidine and the HDAC inhibitor entinostat showed some modest response rates. More impressively, when the patients were anecdotally treated with subsequent therapies, including chemotherapies, immunotherapies (PD-1 checkpoint blockade), and targeted therapies, there appeared to be stronger responses than would be expected for those secondary agents. This leads to the hypothesis that epigenetic drugs may be able to sensitize cancers to other chemotherapeutic, immunotherapeutic, and targeted agents when given concomitantly or sequentially with them. This hypothesis is now being tested in multiple cancer types, including prostate cancer, and it will be interesting to see if such an approach will have utility for treatment of advanced prostate cancer.

Other classes of epigenetic drugs are now being developed, in parallel with our increased understanding of the levels of dysregulation of the targets of those drugs. For histone methylation, given the upregulation of EZH2 in prostate and other cancers, there is significant interest in development of EZH2 inhibitors. Novel agents are being tested in preclinical and early clinical studies. Another exciting class of agents are those targeting the BET bromodomain epigenetic reader proteins involved in “reading” histone acetylation marks to generate activation signals, particularly at regions termed “super-enhancers” that can control the activation of numerous oncogenes including MYC. In prostate cancer preclinical studies, these agents, including the first-in-class drug IQ-1, as well as several new drugs in this class, have shown promising anti-cancer activity. In prostate cancer, the major target of BET bromodomain inhibition for the observed anti-cancer activity is attributed to suppression of androgen receptor expression and signaling. In parallel, there are also efforts to develop inhibitors of the histone acetyltransferases, which would be predicted to have similar effects as the BET bromodomain inhibitors by antagonizing the effects of histone acetylation. Efforts to target other “writers” (e.g., histone methyltransferases), “erasers” (e.g., histone demethylases), and “readers” (e.g., MBD proteins) of many of the epigenetic marks are now underway, and most of these studies are still in early stages. Given the important role of these epigenetic targets in normal as well as cancer cells, whether any of these newer epigenetic agents, including the EZH2 and BET bromodomain inhibitors, will show a
CONCLUSIONS

As our understanding of the epigenetic alterations in human prostate cancer becomes more mature, the field will have many opportunities to translate this understanding to have impact on the clinical management of prostate cancer. Among the different classes of epigenetic alterations in prostate cancer, DNA methylation alterations are the best characterized and are thus the most mature for translation to biomarkers for prostate cancer screening/diagnosis, risk stratification, and treatment/disease burden monitoring. For prostate cancer therapy, particularly in the setting of advanced disease, targeting various components of the epigenetic machinery has shown promise in preclinical studies. Among the most promising of these pharmacological agents to date include the BET bromodomain inhibitors, which have shown activity in castration-resistant prostate cancer preclinical studies. Other classes of agents, including inhibitors of the writers, erasers, and readers of various epigenetic marks are also being investigated. Ongoing and future studies aimed at exploring their utility alone and in combination with androgen deprivation therapy, chemotherapy, immunotherapy, and targeted therapy may yield novel therapeutic strategies for advanced prostate cancer.

COMPETING FINANCIAL INTERESTS

None.

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