Published in final edited form as:

Cancer Res. 2008 November 1; 68(21): 8954–8967. doi:10.1158/0008-5472.CAN-07-6088.

DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity

Srinivasan Yegnasubramanian¹, Michael C. Haffner^{1,7}, Yonggang Zhang^{1,2}, Bora Gurel³, Toby C. Cornish³, Zhijin Wu⁶, Rafael Irizarry⁵, James Morgan^{1,3}, Jessica Hicks^{1,3}, Theodore L. DeWeese^{1,2}, William B. Isaacs^{1,4}, G. Steven Bova³, Angelo M. De Marzo^{1,3,4}, and William G. Nelson^{1,2,3,4}

¹*Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, Maryland, USA*

²*Department of Radiation Oncology, Johns Hopkins University, Baltimore, Maryland, USA*

³*Department of Pathology, Johns Hopkins University, Baltimore, Maryland, USA*

⁴*Brady Urological Institute, Johns Hopkins University, Baltimore, Maryland, USA*

⁵*Department of Biostatistics, School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA*

⁶*Center for Statistical Sciences, Brown University, Providence, Rhode Island 02912, USA*

⁷*Innsbruck Medical University, Christoph-Probst-Platz 1, Innrain 52, A-6020 Innsbruck, Austria*

Abstract

Hypomethylation of CpG dinucleotides in genomic DNA was one of the first somatic epigenetic alterations discovered in human cancers. DNA hypomethylation is postulated to occur very early in almost all human cancers, perhaps facilitating genetic instability and cancer initiation and progression. We therefore examined the nature, extent, and timing of DNA hypomethylation changes in human prostate cancer. Contrary to the prevailing view that global DNA hypomethylation changes occur extremely early in all human cancers, we show that reductions in ⁵meC content in the genome occur very late in prostate cancer progression, appearing at a significant extent only at the stage of metastatic disease. Furthermore, we found that while some LINE1 promoter hypomethylation does occur in primary prostate cancers compared to normal tissues, this LINE1 hypomethylation is significantly more pronounced in metastatic prostate cancer. Next, we carried out a tiered, gene expression microarray and bisulfite genomic sequencing based approach to identify genes that are silenced by CpG island methylation in normal prostate cells but become over-expressed in prostate cancer cells as a result of CpG island hypomethylation. Through this analysis we show that a class of cancer testis antigen genes undergoes CpG island hypomethylation and over-expression in primary prostate cancers, but more so in metastatic prostate cancers. Finally, we show that DNA hypomethylation patterns are quite heterogeneous across different metastatic sites within the same patients. These findings provide evidence that DNA hypomethylation changes occur later in prostate carcinogenesis than the CpG island , and occur heterogeneously during prostate cancer progression and metastatic dissemination.

Correspondence to: Srinivasan Yegnasubramanian; William G. Nelson.

Correspondence: Srinivasan Yegnasubramanian: 1650 Orleans Street, CRB1 Rm 1M53, Baltimore, MD 21231, syegnasu@jhmi.edu; William G. Nelson: 1650 Orleans Street, CRB1 Rm 151, Baltimore, MD 21231, bnelson@jhmi.edu..

Keywords

DNA methylation; hypomethylation; prostate cancer; metastasis; epigenetics; LINE1; tumor heterogeneity; cancer testis antigens

INTRODUCTION

Aberrations in genomic DNA methylation occur almost universally in human cancers. However, the pattern of DNA methylation abnormalities in cancer cells appears paradoxical: compared to normal cells, cancer cells appeared concomitantly hypermethylated at specific CpG island sequences, but hypomethylated at CpGs found in most other sites resulting in a net loss of genomic ⁵meC content (1-3). *Hypermethylation* of promoter CpG islands can silence important tumor-suppressor genes and other genome protection genes (4). *Hypomethylation* of genomic DNA has been associated with increased rates of genomic instability (5,6), perhaps due to decondensation of chromatin into recombination-permissive conformations (7) or due to the activation of retrotransposon elements (8). A few recent studies have linked genomic hypomethylation with increased genetic instability in human cancers (9-12). Another possibility is that CpG island sequences at genes that are normally methylated and silenced in a tissue specific manner (13,14) may become aberrantly hypomethylated, causing inappropriate activation of the corresponding gene in cancer cells. Such aberrations in lineage-specific marks could directly parallel the lack of appropriate differentiation and morphology often seen in cancer cells.

Understanding the relative timing of DNA hypermethylation and hypomethylation changes in cancer is crucial to understanding the importance of these changes in various stages of disease initiation and progression. One prevailing model of epigenetic dysregulation in carcinogenesis and disease progression has held that, on average, reduction of DNA cytosine methylation appears to occur early in carcinogenesis in human cancers, at the stage of pre-malignant lesions or even in aging benign tissues, and that this hypomethylation precedes and perhaps sets the stage for later gene promoter CpG island hypermethylation changes (reviewed in (15)). Some have even hypothesized that age-related decreases in DNA methylation may provide a “field-effect” for cancer initiation, perhaps partially explaining the association of most malignancies with advancing age (9,15).

However, there are hints that prostate cancer may not adhere to this generalized model. An early report showed that reduction in overall ⁵meC content was rare in primary prostate cancers but frequent in metastatic prostate cancer (16). Additionally, LINE1 hypomethylation appeared to occur after hypermethylation of CpG island sequences in the 5' upstream regions of key genes (17). However, these studies examined limited numbers of prostate specimens, did not analyze multiple stages of prostate cancer progression including metastases, or did not thoroughly examine multiple forms of DNA hypomethylation changes. Here, we perform the most comprehensive examination to date of the nature, extent, and timing of DNA hypomethylation changes in prostate cancer disease progression. Specifically, we hypothesize that such DNA hypomethylation changes can manifest in at least three major ways: i) reduction in genomic ⁵meC content, ii) decreased methylation of repetitive sequences, such as *LINE1* elements, and iii) hypomethylation of normally methylated promoter CpG island sequences leading to overexpression of the associated genes. We show that these hypomethylation changes occurred late in prostate cancer disease progression, manifesting most prominently at the stage of metastatic disease. Furthermore, compared to CpG island hypermethylation changes, DNA hypomethylation changes were heterogeneous across different metastatic deposits within the same cases. Taken together, these data suggest that, contrary to the prevailing model of epigenetic dysregulation, DNA hypomethylation occurs late in prostate

cancer disease progression and is unlikely to be involved in prostate cancer initiation and more likely to be involved in the formation and propagation of metastases.

MATERIALS AND METHODS

Cell culture, DNA, and RNA extraction

Primary cultures of normal prostate epithelial cells (PrECs), two prostate stromal cell models (4ST, and 1S), and 6 prostate cancer cell lines (LNCaP, C4-2B, PC-3, DU-145, LAPC-4, CWR22Rv1) were propagated in tissue culture as described previously (18). Cells were allowed to reach 80% confluence in tissue culture flasks. Whole RNA and genomic DNA were isolated using the RNeasy kit (Qiagen, Valencia, CA) and the DNeasy Tissue kit (Qiagen) respectively by following the manufacturer's protocols. RNA and DNA were quantitated by 260 nm λ UV absorbance using a standard spectrophotometer.

Tissue specimens, tissue microarrays, and DNA isolation

Tissues from primary prostate cancer tissues from 76 men undergoing radical prostatectomy, tumor-adjacent benign tissues from 12 of these men, multiple anatomically distinct metastatic prostate lesions from 32 men undergoing autopsy after dying from hormone refractory metastatic cancer, lymph node metastases from 8 men undergoing radical prostatectomy for clinically presumed localized prostate cancer (the radical prostatectomy was aborted in these men due to the discovery of metastatic lymph nodes), and normal prostate specimens from 13 brain-dead organ donors with no evidence of prostate disease, were obtained and subjected to DNA isolation as previously described (18). The tumor-adjacent benign prostate tissues included 7 specimens containing extensive regions of PIA, 5 specimens containing extensive regions of PIN of which one specimen also had extensive PIA, and one specimen with neither PIA nor PIN. See Supplementary Table S1 for a summary of DNA specimens available from tissue specimens and average age and age range of each DNA specimen group. Two hundred and twenty core tissue microarrays were constructed from formalin-fixed paraffin-embedded tissue blocks as previously described (19). All studies with human specimens were approved by our institutional review board.

Quantitation of genomic ⁵meC content by High Pressure Liquid Chromatography-Tandem Mass Spectrometry (LC-MC/MS)

The overall 5-methyl-2'-deoxycytidine (⁵meC) content (as percentage of total 2'-deoxycytidine (2dC) content) in genomic and control DNAs was determined by an HPLC/Tandem Mass Spectrometry (LC/MS/MS) procedure as described elsewhere (20,21), with minor modifications. Please see Supplementary Materials for a detailed description.

Gene expression microarray analysis, bioinformatics, and statistical analysis of methylation data

10 μ g of total RNA from PrEC, 4ST, 1S, LNCaP, PC-3, DU-145, LAPC-4, C4-2B, and CWR22Rv1 cells were processed, labeled, and hybridized to hgU133A whole genome gene expression microarrays (Affymetrix, Santa Clara, CA) according to the manufacturer's protocols. The Bar Code gene expression microarray analysis (22), with minor modifications, was used to identify genes that were expressed in the cancer cell lines with high confidence, but not expressed in the PrEC, 4ST, and 1S normal cells.. Please see Supplementary Materials for a detailed description of microarray analysis procedures, bioinformatics analysis of the distribution of CpG dinucleotides in the human genome, and statistical methods used to analyze methylation data.

Bisulfite genomic sequencing

Bisulfite genomic sequencing was carried out on genomic DNA samples that were bisulfite converted using the EZ DNA methylation kit (Zymo Research, Orange, CA) as previously described (23). Primers and annealing temperatures used to amplify bisulfite converted genomic DNA are listed in Supplementary Table S3.

COMPARE methylation assay

A variation of the previously described COMPARE-MS assay (23) termed the COMPARE (Combination Of Methylated-DNA Precipitation And Restriction Enzyme digestion) assay, was used to determine the degree of CpG island methylation at candidate hypomethylated genes and at *LINE1* consensus promoter sequences in the prostate cell line and tissue samples. The COMPARE assay is identical to the COMPARE-MS assay except that the methylation-sensitive restriction enzyme was omitted (23). A detailed description of the COMPARE assay can be found in Supplementary Materials with PCR primers listed in Supplementary Table S4.

Immunohistochemistry and analysis

Immunohistochemical staining was carried out on deparaffinized and rehydrated tissue sections from formalin-fixed paraffin-embedded blocks or TMAs. Immunohistochemistry for NY-ESO-1 (encoded by *CTAG1B* gene) and MAGEA1 was done with the EnVision+ kit (DakoCytomation, Carpinteria, CA). For NY-ESO-1, slides were steamed for 20 minutes in 1x Citrate Unmasking Solution (Vector Labs, Burlingame, CA) for antigen retrieval and then incubated with anti-NY-ESO-1 primary antibodies (1:100 dilution; Zymed, South San Francisco, CA) for 45 minutes at room temperature. For MAGEA1, slides were steamed in 1x EDTA buffer (Zymed) for 40 minutes and stained with anti-MAGEA1 primary antibody (1:100 dilution; Zymed) at room temperature for 45 minutes. Immunohistochemistry with anti-^{5meC} antibody was done with the PowerVision+ Detection System (Immunovision Technologies, Norwell, MA). Antigen retrieval was accomplished by steaming slides for 20 minutes in Citrate Unmasking Solution and then treating with 3.5 N HCl for 15 min. Slides were then stained with anti-^{5meC} primary antibody (1:4000 dilution; Calbiochem, San Diego, CA). The TMA slides were digitally scanned and the immunohistochemical staining was analyzed using the FrIDA image analysis tool (24); <http://bui2.win.ad.jhu.edu/frida/>). See Supplementary Materials for a detailed description of evaluation of immunohistochemical staining and quantitative image analysis using FrIDA.

RESULTS AND DISCUSSION

Genomic ^{5meC} content is reduced in metastatic but not primary prostate cancers

DNA methylation occurs predominantly in CpG dinucleotides in adult somatic tissues in vertebrate genomes. Since CpG dinucleotides are known to be non-uniformly distributed in the human genome (25-27), we examined their distribution in the reference human genome sequence (Supplementary Fig. S1A). Approximately 4.85% of all cytosines occur in CpG dinucleotides in the human male genome. Of these CpGs, ~23% occur in CpG islands, in which 1/5 - 1/4 of cytosines occur in the context of CpG dinucleotides. The remaining ~77% of CpGs occur very sparsely throughout the genome with ~1/20 cytosines occurring in CpG sequences. Therefore, measurements of overall genomic ^{5meC} content most likely reflect the degree of methylation of these sparsely-spaced CpG dinucleotides in the human genome.

Using a highly sensitive LC-MS/MS approach, we measured overall genomic ^{5meC} content as a fraction of total genomic 2dC content (^{5meC} + 2dC) in a collection of prostate cell lines and control specimens (Fig. 1A). This method was capable of accurately detecting 2 fmol of ^{5meC} and 2dC, showed linearity over a broad dynamic range spanning 2 to 3 orders of magnitude,

and allowed robust measurement of overall 5meC content from as little as 1 ng of genomic DNA (data not shown). This method was highly accurate when tested with control oligonucleotides containing 0% (UM-Oligo; data not shown) and 80% (M-Oligo 80) actual 5meC content.

Furthermore, the M.SssI-treated WBC genomic DNA, in which all CpG dinucleotides were enzymatically methylated *in vitro*, had a 5meC content of ~4.9 %, representing the maximal amount of CpG methylation possible in a normal human genomic DNA specimen. Note that this is also an estimate of the percentage of cytosines in CpG dinucleotides in the human genome and is in close agreement with the *in silico* analysis of CpG content in the human genome, validating this assay for analysis of genomic DNA samples. Untreated normal WBCs and normal prostate epithelial (PrEC) and prostate stromal (4ST) cells had 5meC contents between ~3.5% and 4.0% suggesting that approximately 70 - 80% of all CpGs were methylated in these nonmalignant cells. In comparison to these normal cells, 5 of the 6 prostate cancer cell lines (LNCaP, C4-2B, LAPC-4, CWR22Rv1, and PC-3) had significantly reduced 5meC content (2.0% - 2.9%) with only DU-145 showing a 5meC content (3.9%) that was not reduced.

We next examined genomic DNA from a large collection of prostate tissues (Fig. 1B). The 5meC content of benign prostate tissues resembled that of the WBC, PrEC, and 4ST cells. Surprisingly, compared to benign prostate specimens, overall 5meC content was reduced in the metastatic ($p < 0.0001$), but not primary prostate cancers or tumor adjacent PIA/PIN/normal tissues. The 5meC content of these metastases resembled that of the prostate cancer cell lines, which were originally derived from prostate cancer metastases (28-31). Furthermore, there was a statistically significant trend in 5meC content with primary cancer > hormone naïve lymph node metastases > hormone refractory autopsy metastases ($p\text{-trend} < 0.0001$). Immunohistochemical analysis and quantitative image analysis of anti- 5meC -antibody stained tissue microarrays (TMAs) containing 220 cores with an assortment of organ donor normal prostate tissues, primary prostate cancer tissues from radical prostatectomy, and metastatic prostate cancers from a rapid autopsy series, confirmed that reductions in 5meC content occurred in metastatic, but not primary prostate cancer tissues (Fig. 1C, D). Taken together, these findings suggest that overall decreases in genomic 5meC content occur late in prostate cancer progression, at the stage of metastatic disease. This is in stark contrast with the conventional view that reductions in overall 5meC content generally occur early in carcinogenesis and disease progression.

Degree of *LINE1* promoter methylation is reduced in metastatic prostate cancer compared to primary prostate cancer and benign prostate

Repetitive elements constitute nearly half of all sequences and half of the CpG content in the human genome (Supplementary Fig. S1B)(26, 27). LINE1 (L1) elements are the largest class of repetitive elements in the human genome, constituting approximately 16% of all sequences and ~9.5% of all CpGs. Of particular interest, LINE1 elements possess an internal promoter, which contains a high density of CpG dinucleotides that can meet criteria for a CpG island (32). The LINE1 promoter CpG island is typically methylated (33), and its hypomethylation can lead to transcriptional activation of intact LINE1 elements, induce retrotransposition, and facilitate genetic instability (34, 35). The vast majority of LINE1 elements, however, are truncated or highly degenerate, lack the promoter sequence, and are not capable of active retrotransposition (26, 36). Since they constitute a small fraction of all CpGs in the human genome, LINE1 promoters could become hypomethylated even without significant overall reductions in genomic 5meC content. Therefore, we investigated the possibility that LINE1 promoter CpG islands may become hypomethylated during the initiation and progression of prostate cancer.

We used a derivative of the recently described COMPARE-MS assay (23), which takes advantage of the high affinity and selectivity of the methyl-binding domain fragment of MBD2 (MBD2-MBD) in binding symmetrically methylated DNA, to assess the extent of LINE1 methylation in prostate specimens. In the modified assay (Fig. 2A), which we term COMPARE, each genomic DNA specimen was split into an untreated fraction and an M.SssI-treated fraction, in which all CpGs were fully methylated. These samples were fragmented by restriction enzyme digestion and subjected to enrichment for methylated DNA fragments by capture with MBD2-MBD polypeptides immobilized on magnetic beads. Enriched methylated LINE1 promoter fragments were then detected by real-time PCR for the consensus LINE1 promoter (32). The Methylation Index (MI), which is the ratio of the signal from the untreated fraction to the M.SssI treated fraction for each specimen, gives a measure of the percentage of input copies that were methylated at a given locus of interest. In comparison with the original assay, omission of the methylation-sensitive restriction enzyme in this COMPARE assay allowed more stringent quantitation of hypomethylation since only sequences that are sufficiently undermethylated to escape capture with the MBD2-MBD would appear hypomethylated. The COMPARE assay exhibited a linear dynamic range spanning >3 orders of magnitude, and could be used to accurately measure DNA methylation from as little as 32 pg of genomic DNA (23). Furthermore, the COMPARE LINE1 promoter hypomethylation assay showed strong concordance with results of bisulfite genome sequencing of LINE1 promoters in reference samples, showing that this assay had a linear dynamic range from no LINE1 methylation to complete LINE1 methylation (Supplementary Figure S7).

Application of the COMPARE LINE1 hypomethylation assay revealed that prostate cancer cell lines had less LINE1 promoter methylation than the normal WBC, PrEC, and 4ST cells, which showed approximately 80 - 90% methylation of input LINE1 promoter sequences. In particular, the LNCaP and C4-2B cell lines had less than half the LINE1 promoter methylation levels as the normal cells (Fig. 2B). The benign prostate tissues from organ donors, and the PIA/PIN/tumor-adjacent benign prostate tissues exhibited high levels of LINE1 methylation similar to the WBC, PrEC, and 4ST genomic DNA specimens (Fig. 2C). In comparison with these benign specimens, the primary prostate cancers had decreased LINE1 methylation even though they did not exhibit a significant reduction in overall genomic ⁵meC content. This finding is in agreement with previous reports showing decreased LINE1 element methylation in primary prostate cancers (17). It is possible that DNA hypermethylation changes in the primary prostate cancers sufficiently compensate for the decreased LINE1 methylation so that there is no net reduction in the genomic ⁵meC content. Finally, metastatic prostate cancers had even lower LINE1 methylation than the primary prostate cancers. For LINE1 promoter methylation also, there was a statistically significant trend with primary cancers > hormone naïve lymph node metastases > hormone refractory autopsy metastases ($p < 0.0032$). These findings suggest that LINE1 hypomethylation occurs to some extent in primary prostate cancers compared to normal tissues, but is more pronounced in metastatic prostate cancer.

A tiered gene-expression microarray and bisulfite sequencing based approach to identify gene-specific CpG island hypomethylation changes in prostate cancer cells

Ever since the finding that DNA methyltransferase inhibitors can induce differentiation changes in cells (37), the notion that DNA methylation patterns are partly responsible for maintaining the differentiated phenotype has been very attractive. In support of this notion, recent studies have identified tissue differentially methylated (TDM) genes that are selectively methylated in certain organs and cell types but not in others within individuals (13). More recently, a link to carcinogenesis was established by studies finding that some genes that are normally methylated and silenced in specific tissues can become hypomethylated and over-expressed in cancers arising in that tissue (38-40).

Here, we used a tiered genome-wide gene expression microarray and bisulfite sequencing based strategy to identify normally silenced and methylated genes that become expressed in prostate cancer cells due to CpG island hypomethylation. The central premise was that normally methylated genes that become hypomethylated in cancers may be identifiable as those genes that are absolutely unexpressed in normal cells but are expressed significantly in cancer cells. We therefore carried out gene expression microarray experiments on normal prostate epithelial and stromal cells (PrEC, 4ST, 1S) and on 6 prostate cancer cell lines (LNCaP, C4-2B, LAPC-4, CWR22Rv1, PC-3, DU-145) using the Affymetrix hgU133A whole genome expression array platform. Next, we applied the recently developed “Bar Code” gene expression microarray analysis (22) to identify genes that were likely to be unexpressed in the normal cells, and likely to be robustly expressed in at least one of the 6 cancer cell lines. Unlike most conventional microarray analytical methods, which are best suited to identifying relative patterns of expression (i.e. over- or under- expressed genes compared to a reference), Bar Code microarray analysis was designed to identify absolute patterns of expression (i.e. classification of a gene transcript as being absent or present). The Bar Code microarray analysis was recently shown to be a powerful classification tool (22).

Applying this Bar Code microarray analysis method with stringent thresholds for our set of normal and malignant prostate cell systems, we identified 168 probe sets that showed high confidence of expression in at least one prostate cancer cell line, while showing high confidence of absence of expression in the normal prostate epithelial and stromal cells (Fig. 3, Supplementary Table S2). Removal of redundant probe sets targeting the same gene and selection of genes with 5' CpG islands gave a total of 120 unique genes with 5' CpG islands that showed absolute expression in the cancer cells and lack of expression in the normal cells - a pattern consistent with cancer-specific gene CpG island hypomethylation. We noted that a surprisingly large fraction (18%) of these genes could be classified as sex-chromosome associated cancer-testis antigen (CTA) genes, so-named because of their expression in normal testis and many cancers, but not in other normal tissues (41). Since many CTA genes have been previously found to become hypomethylated in cancers, we selected all of these genes (21 total) as well as another 10 randomly selected genes from a variety of functional categories, and successfully designed bisulfite sequencing assays for 22 genes, including 14 CTA genes and 8 other genes. Interestingly, multiple CTA genes often shared identical 5' upstream CpG island sequences, both in direct and inverted repeat configurations. In some cases, the associated 5' upstream CpG island was repeated >10 times across the X chromosome in the reference genome. All genes that were found to have methylated 5' upstream CpG islands in the normal PrEC, 4ST, and/or WBC samples were sequenced in all of the 6 prostate cancer cell lines to see if they became hypomethylated at these CpG islands.

By this analysis, a total of 14 genes (CTAG1B, CTAG2, GAGE2, GAGE3, GAGE4, GAGE6, GAGE7, GAGE7B, MAGEA1, MAGEA3, MAGEA6, MAGEA12, PAGE1, TSPY1), containing 7 unique 5' upstream CpG island sequences, were found to be hypomethylated in at least one of the cancer cell lines compared to the normal cells (Fig. 4, Supplementary Fig. S2). Expression of the majority of these hypomethylated CTA genes showed a strong inverse correlation with methylation levels (Fig. 4, Supplementary Fig. S8), suggesting that hypomethylation of these genes is strongly associated with their over-expression in cancer cells. The only significant exceptions to this general trend were the GAGE genes, which all shared a single CpG island that was repeated >10 times on the X chromosome. Therefore, it is likely that this CpG island is either not involved in the regulation of these genes, or plays a very complex role that is not easily deciphered. Surprisingly, all of these candidate hypomethylated and over-expressed genes belonged to the group of CTA genes, while none of the non-CTA genes tested showed any methylation in the normal WBC and PrEC cells, and therefore, by definition, could not be hypomethylated in the cancer cell lines since they were already unmethylated in the normal cells (Supplementary Fig. S3). It should be noted that these

studies were not intended to identify all hypomethylated genes since there are many other candidates from the original list that were not tested and use of less stringent thresholds would have identified many other candidates. Nonetheless, identification of this set of hypomethylated and over-expressed genes in the prostate cancer cell lines allowed subsequent analysis of the nature, extent, and timing of these representative gene CpG island hypomethylation changes in prostate cancer tissues.

We designed high-throughput COMPARE assays to assess the extent of CpG island hypomethylation at these candidate genes in prostate cancers. Results from these COMPARE assays were highly correlated with results from bisulfite sequencing in the prostate cell lines (Fig. 4, Supplementary Fig. S8), suggesting that COMPARE was a good surrogate for bisulfite sequencing analysis, with the advantage that it was more amenable to high-throughput analysis. Application of these assays to prostate tissues revealed that, compared to the organ donor and tumor-adjacent benign PIA/PIN/normal prostate tissues, nearly all of the 7 candidate CpG islands were hypomethylated in the primary but more so in the metastatic prostate cancers (Fig. 5A). Additionally, for six of the seven CpG islands tested, there was a statistically significant trend in the extent of methylation, with primary cancer > hormone naïve lymph node metastases > hormone refractory autopsy metastases. Data from microarray studies in the Oncomine database (42) showed that these hypomethylated genes were, in general, over-expressed at the mRNA level in prostate cancers compared to benign prostate and that the over-expression was often more pronounced in metastatic prostate cancers than in primary prostate cancers (Supplementary Fig. S4). Furthermore, immunohistochemical (IHC) analysis of NY-ESO-1, encoded by the *CTAG1B* gene, and MAGEA1 in the prostate cancer cell lines showed that these proteins were only expressed in those cell lines that had significant hypomethylation at the 5' CpG island of their corresponding gene (Fig. 5B, Supplementary Fig. S5). IHC analysis of NY-ESO-1 in a prostate TMA containing 116 metastatic lesions, 26 primary cancers, and 19 benign prostate tissues showed that control testis tissues and a subset of metastases (13%) showed robust staining, but that none of the primary cancers or benign prostate tissues showed any staining (Fig. 5C). IHC analysis of MAGEA1 in prostate TMAs showed weak to moderate positive staining in approximately 3 - 5% of primary and metastatic prostate cancers, and no staining in benign prostate tissues (data not shown). These findings are in general agreement with previous studies examining the expression pattern of these CTAs in the prostate (43). The significantly lower frequency of protein staining compared to the frequency of DNA hypomethylation and mRNA expression in the metastatic and primary cancers may be an indication that mRNA expression and/or CpG island hypomethylation is not sufficient for protein expression for these genes in prostate cancer tissues. Alternatively, it is possible that the low degree of staining in the prostate cancer tissues compared to the cell lines may reflect a limitation in the ability to detect these proteins by IHC in prostate tissue sections in which antigen preservation and presentation may have been less robust than in freshly prepared and fixed cell line specimens. Evaluation of commercially available antibodies for proteins encoded by the other hypomethylated genes, including MAGEA3, MAGEA6, MAGEA12, and TSPY1, showed that IHC analyses with these antibodies were highly sensitive to tissue preparation and fixation conditions, often showed high non-specific signal and low dynamic range, and were therefore not well suited for IHC analysis of our TMAs (data not shown).

These results suggest that while some gene specific CpG island hypomethylation does occur in primary prostate cancers, these DNA hypomethylation changes, like LINE1 promoter hypomethylation and overall ⁵meC content reductions, are most prominent in the metastatic prostate cancers.

DNA hypomethylation changes are more heterogeneous across different anatomically distinct metastatic deposits within the same patients than characteristic CpG island hypermethylation changes

Yegnasubramanian et al. previously showed that nearly all the anatomically distinct metastatic deposits from a given subject showed very similar CpG island hypermethylation patterns, suggesting that these changes were present in the primary cancer and were closely maintained during and after metastatic dissemination (18). Here, we examined whether DNA hypomethylation changes were also closely maintained during the process of metastatic dissemination by analyzing the same metastatic deposits that were examined in the previous study of CpG island hypermethylation. Compared to the CpG island hypermethylation changes, DNA hypomethylation changes were much more heterogeneous across different anatomically distinct metastatic deposits from the same subject (Fig. 6A). To confirm this visual observation, we used linear regression models to fit the CpG island hypermethylation and CTA gene hypomethylation data using patients, DNA methylation markers, and patient-marker interactions as independent variables. In addition, we fit the LINE1 promoter methylation and 5mC content data using patients as the independent variable. All of these models adequately fit the data, accounting for ~71% and ~91% of the variance in the CTA gene hypomethylation and CpG island hypermethylation data respectively, and for ~81% and ~67% of the LINE1 promoter hypomethylation and 5mC content data respectively. Examination of the residual variances, which represent the variance across sites within each patient or patient-marker combination, revealed that the variance in the hypomethylation data across sites within each patient was significantly higher than the variance in the CpG island hypermethylation data by the F-test (5mC content: 2.1 fold higher variance, $p < 10^{-5}$; LINE1 promoter methylation: 1.7 fold higher variance, $p < 10^{-3}$; CTA gene hypomethylation: 2.1 fold higher variance, $p < 10^{-10}$). Additionally, the pattern of 5mC immunohistochemical staining in these metastatic deposits was very heterogeneous across metastatic deposits from the same subject, and even appeared to be quite focal and heterogeneous within a single metastatic deposit in some samples (Fig. 6B). Applying the same type of regression analysis, the variance in the 5mC IHC data across sites within patients was >3.5 fold higher than that in the CpG island hypermethylation data ($p < 10^{-10}$). Finally, consistent with the pattern of hypomethylation in the metastatic lesions, immunohistochemical staining of these metastatic deposits for NY-ESO-1 showed that its expression was also heterogeneous across different metastatic deposits within the same patient and often showed focal heterogeneous expression within a single metastatic deposit (Fig. 6C). This CTA gene expression heterogeneity is consistent with that observed previously even in primary cancers (41), and suggests that NY-ESO-1 expressing cells from the primary cancers were unlikely to have been clonally selected for during the process of metastatic dissemination since the metastases also show considerable heterogeneity in their expression of NY-ESO-1. Additionally, DNA hypomethylation changes, like the CpG island hypermethylation changes, did not show any site specific patterns, but were rather heterogeneous across patients within each site category (Supplementary Fig. S6). These data suggest that DNA hypomethylation changes occur most prominently during or after metastatic dissemination, and contribute to tumor heterogeneity in metastatic prostate cancer.

A model of DNA methylation changes in prostate cancer initiation and progression and its implications for disease pathogenesis and treatment

Several studies have previously shown that CpG island hypermethylation changes occur nearly universally in prostate cancer, often arise early in prostate cancer initiation and progression, and, once they arise, are closely maintained throughout disease progression and metastatic dissemination (18,44). In contrast, here we show that DNA hypomethylation changes occur late in prostate cancer progression, occurring most frequently in metastatic lesions, and are often heterogeneous across different metastatic deposits within the same subjects. Since others have reported age-related changes in DNA methylation (e.g.,(45,46)), we examined whether

the hypomethylation changes between groups observed in this study were independent of age. These DNA hypomethylation changes were not simply a function of increasing age in the men with advanced prostate cancer since nearly all statistically significant differences in 5meC content, LINE1 hypomethylation, and CTA gene hypomethylation between different tissue categories remained significant even after adjusting for age in multivariate regression models.

Interestingly, the tumor-adjacent benign prostate tissues containing areas of PIN and/or PIA often contained low levels of CpG island hypermethylation changes (18,23), but did not have any apparent reductions in 5meC content or hypomethylation of LINE1 and CTA gene promoters. Additionally, while the CpG island hypermethylation changes examined previously occurred nearly universally in primary prostate cancer, only a fraction of these primary cancers showed decreases in LINE1 and CTA gene promoter methylation. These findings suggest that these hypomethylation changes occur later in prostate cancer progression than the CpG island hypermethylation changes and may indicate that DNA hypomethylation changes are not important in prostate cancer initiation but may be more important in the formation/progression of lethal metastatic prostate cancer. This is in stark contrast to findings in other cancers where DNA hypomethylation reportedly occurs early in carcinogenesis and contributes to initiation of the neoplastic phenotype, occurring frequently and prominently in precancerous lesions of organs such as lung and colon (9,15).

Instead, the findings here suggest that most of the DNA hypomethylation changes examined in this study were highly correlated with lethal metastatic prostate cancer. Interestingly, while significant 5meC content reductions occurred only at the stage of metastatic prostate cancer, LINE1 promoter hypomethylation and CTA gene hypomethylation appeared first in primary prostate cancers, and became more pronounced in metastatic prostate cancers. While these observations may suggest that LINE1 hypomethylation or CTA gene hypomethylation in primary cancers may lead to a selective advantage for the development of metastatic disease, two major findings in this study provide some evidence against this hypothesis. First, while CpG island hypermethylation changes were uniformly maintained across multiple metastases from the same individual, indicating that these arose prior to metastatic dissemination, DNA hypomethylation changes were much more variable across different metastases from the same individual, suggesting that these changes commonly arose after metastatic dissemination. Second, while various CpG island hypermethylation changes were highly associated with pathological stage, grade, and risk of recurrence (18), none of the DNA hypomethylation changes in primary cancers observed here were associated with pathological grade, stage, or recurrent disease (data not shown). These findings suggest that, although the LINE1 and CTA promoter hypomethylation sometimes occur in the primary cancers, these hypomethylation changes do not confer an increased risk for aggressive disease, and are not necessarily selected for and maintained during metastatic dissemination. It is also interesting to note that the primary cancers had a statistically significant increase in the global 5meC content compared to the organ donor normal prostate specimens, despite having decreases in LINE1 promoter and CTA gene methylation. This suggests that hypomethylation changes occurring in LINE1 promoter regions and CTA gene 5' upstream regions in these primary cancers are offset and sometimes overbalanced by hypermethylation changes in other portions of the genome, likely at numerous CpG islands.

These observations imply that the DNA hypomethylation changes occur most consistently during or after metastatic dissemination, at which point they may even contribute to the maintenance and propagation of the metastatic lesions. Several recent reports have shown that increased hypomethylation is highly correlated with genetic instability in human malignancies (9-11). The increased hypomethylation observed in the metastatic prostate cancer lesions may contribute to the high number and prevalence of chromosomal aberrations previously reported in metastatic prostate cancers compared to primary cancers (47). One recent report synthesizing

data from 41 studies analyzing 872 cancer specimens showed that advanced prostate cancer specimens show a significantly higher frequency of chromosomal abnormalities than primary prostate cancer specimens (48). Consistent with this hypothesis is the finding that mice with hypomorphic DNMT enzymes are prone to genetic instability and lymphomagenesis (49).

The previous findings regarding CpG island hypermethylation alterations in prostate cancer highlighted a fundamental paradox: there was enough dysregulation of the DNA methylation machinery to produce the initial CpG island hypermethylation changes, and yet there was enough fidelity in the DNA methylation machinery so that a specific set of CpG island hypermethylation changes could be closely maintained throughout disease progression even through metastasis. These observations were consistent with the notion that there is an early dysregulation of the *de novo* DNA methylation machinery, but that the maintenance DNA methylation machinery remains intact allowing for maintenance of these changes throughout disease progression. Yet, the data obtained here suggests that this cannot be completely accurate. Indeed, there appears to be a progressive dysregulation of maintenance methylation, manifesting most prominently late in disease and causing reductions in overall ⁵mC content, in *LINE1* methylation, and in gene-specific CpG island methylation. These data suggest a revised model for dysregulation of DNA methylation in prostate cancer: increased *de novo* methylation early in prostate carcinogenesis to produce coordinate CpG island hypermethylation changes, with progressive loss of maintenance methylation in the midst of selective pressures to maintain hypermethylation of CpG islands in the 5' upstream region of several key genes (Fig. 6D). Such a model could explain the early and frequent appearance of several CpG island hypermethylation changes in prostate cancer, the ability to maintain these changes at key CpG islands during disease progression, and the loss of overall ⁵mC content, *LINE1* methylation, and gene-specific CpG island methylation occurring late in prostate cancer progression.

Tumor cell heterogeneity, particularly in the expression and function of therapeutic targets, is a common feature in advanced cancers that poses major challenges in the development of effective treatments. Here, we show that DNA hypomethylation is a significant source of tumor heterogeneity in metastatic prostate cancer and may contribute to development of therapeutic resistance. In particular, we show that gene-specific DNA hypomethylation changes can cause heterogeneous over-expression of a series of cancer-testis antigen genes, many of which are currently being evaluated as targets for immunotherapy (41,50). Data from recent clinical trials have demonstrated regression of tumors when patients were treated with immunotherapies targeted to these CTA antigens (51,52). However, since there is significant heterogeneity in the DNA hypomethylation and CTA gene expression patterns from tumor to tumor in metastatic prostate cancer, vaccine strategies directed against a single CTA target may be of limited utility, unless these strategies can also produce more promiscuous immune responses to eradicate non-expressing tumor cells, as recently described (53). Based on the heterogeneity of DNA hypomethylation and associated gene over-expression shown in this study, further studies to test the potential of vaccine strategies directed against multiple CTA gene products in combination or use of vaccines containing multiple heterogeneous whole tumor cell models may yield new therapies for advanced prostate cancer (54).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We would like to thank Jeanne Kowalski and Amanda Blackford for providing advice with regards to analysis of the gene expression microarray data, Kristen Lecksell for assistance in scanning TMAs, and the Johns Hopkins TMA facility for preparation and processing of TMAs.

This work was supported by grants from the NIH/NCI (CA070196, CA113374, CA58236, CA92234), and the Prostate Cancer Foundation.

REFERENCES

1. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983;301:89–92. [PubMed: 6185846]
2. Gama-Sosa MA, Slagel VA, Trewyn RW, et al. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res* 1983;11:6883–94. [PubMed: 6314264]
3. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;61:3225–9. [PubMed: 11309270]
4. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999;21:163–7. [PubMed: 9988266]
5. Karpf AR, Matsui S. Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. *Cancer Res* 2005;65:8635–9. [PubMed: 16204030]
6. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 2003;300:455. [PubMed: 12702868]
7. Tuck-Muller CM, Narayan A, Tsien F, et al. DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. *Cytogenetics and cell genetics* 2000;89:121–8. [PubMed: 10894953]
8. Steinhoff C, Schulz WA. Transcriptional regulation of the human LINE-1 retrotransposon L1.2B. *Mol Genet Genomics* 2003;270:394–402. [PubMed: 14530963]
9. Suzuki K, Suzuki I, Leodolter A, et al. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. *Cancer Cell* 2006;9:199–207. [PubMed: 16530704]
10. Cadieux B, Ching TT, Vandenberg SR, Costello JF. Genome-wide Hypomethylation in Human Glioblastomas Associated with Specific Copy Number Alteration, Methylenetetrahydrofolate Reductase Allele Status, and Increased Proliferation. *Cancer Res* 2006;66:8469–76. [PubMed: 16951158]
11. Rodriguez J, Frigola J, Vendrell E, et al. Chromosomal Instability Correlates with Genome-wide DNA Demethylation in Human Primary Colorectal Cancers. *Cancer Res* 2006;66:8462–9468. [PubMed: 16951157]
12. Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. *Biochim Biophys Acta* 2007;1775:138–62. [PubMed: 17045745]
13. Song F, Smith JF, Kimura MT, et al. Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. *Proc Natl Acad Sci U S A* 2005;102:3336–41. [PubMed: 15728362]
14. Kitamura E, Igarashi J, Morohashi A, et al. Analysis of tissue-specific differentially methylated regions (TDMs) in humans. *Genomics* 2007;89:326–37. [PubMed: 17188838]
15. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006;7:21–33. [PubMed: 16369569]
16. Bedford MT, van Helden PD. Hypomethylation of DNA in pathological conditions of the human prostate. *Cancer Res* 1987;47:5274–6. [PubMed: 2443238]
17. Flori AR, Steinhoff C, Muller M, et al. Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. *Br J Cancer* 2004;91:985–94. [PubMed: 15292941]
18. Yegnasubramanian S, Kowalski J, Gonzalgo ML, et al. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res* 2004;64:1975–86. [PubMed: 15026333]
19. Faith DA, Isaacs WB, Morgan JD, et al. Trefoil factor 3 overexpression in prostatic carcinoma: prognostic importance using tissue microarrays. *Prostate* 2004;61:215–27. [PubMed: 15368473]
20. Agoston AT, Argani P, Yegnasubramanian S, et al. Increased protein stability causes DNA methyltransferase 1 dysregulation in breast cancer. *J Biol Chem* 2005;280:18302–10. [PubMed: 15755728]
21. Lee BH, Yegnasubramanian S, Lin X, Nelson WG. Procainamide is a specific inhibitor of DNA methyltransferase 1. *J Biol Chem*. 2005(In Press)
22. Zilliox MJ, Irizarry RA. A gene expression bar code for microarray data. *Nature Methods*. 2007In Press

23. Yegnasubramanian S, Lin X, Haffner MC, DeMarzo AM, Nelson WG. Combination of methylated-DNA precipitation and methylation-sensitive restriction enzymes (COMPARE-MS) for the rapid, sensitive and quantitative detection of DNA methylation. *Nucleic Acids Res* 2006;34:e19. [PubMed: 16473842]
24. Cornish T, Morgan JD, Gurel B, De Marzo A. FRIDA: An Open Source Framework for Image Dataset Analysis. *Archives of Pathology and Laboratory Medicine*. 2007In Press
25. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature* 1986;321:209–13. [PubMed: 2423876]
26. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature* 2001;409:860–921. [PubMed: 11237011]
27. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science* 2001;291:1304–51. [PubMed: 11181995]
28. Webber MM, Bello D, Quader S. Immortalized and tumorigenic adult human prostatic epithelial cell lines: characteristics and applications Part 2. Tumorigenic cell lines. *Prostate* 1997;30:58–64. [PubMed: 9018337]
29. Klein KA, Reiter RE, Redula J, et al. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nat Med* 1997;3:402–8. [PubMed: 9095173]
30. Sramkoski RM, Pretlow TG 2nd, Giaconia JM, et al. A new human prostate carcinoma cell line, 22Rv1. *In vitro cellular & developmental biology* 1999;35:403–9.
31. Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. *Int J Cancer* 1994;57:406–12. [PubMed: 8169003]
32. Crowther PJ, Doherty JP, Linsenmeyer ME, Williamson MR, Woodcock DM. Revised genomic consensus for the hypermethylated CpG island region of the human L1 transposon and integration sites of full length L1 elements from recombinant clones made using methylation-tolerant host strains. *Nucleic Acids Res* 1991;19:2395–401. [PubMed: 1710354]
33. Nur I, Pascale E, Furano AV. The left end of rat L1 (L1Rn, long interspersed repeated) DNA which is a CpG island can function as a promoter. *Nucleic Acids Res* 1988;16:9233–51. [PubMed: 2459662]
34. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* 1997;13:335–40. [PubMed: 9260521]
35. Symer DE, Connelly C, Szak ST, et al. Human L1 retrotransposition is associated with genetic instability in vivo. *Cell* 2002;110:327–38. [PubMed: 12176320]
36. Szak ST, Pickeral OK, Makalowski W, Boguski MS, Landsman D, Boeke JD. Molecular archeology of L1 insertions in the human genome. *Genome biology* 2002;3research0052
37. Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 1980;20:85–93. [PubMed: 6156004]
38. Akiyama Y, Maesawa C, Ogasawara S, Terashima M, Masuda T. Cell-type-specific repression of the maspin gene is disrupted frequently by demethylation at the promoter region in gastric intestinal metaplasia and cancer cells. *Am J Pathol* 2003;163:1911–9. [PubMed: 14578190]
39. Gupta A, Godwin AK, Vanderveer L, Lu A, Liu J. Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res* 2003;63:664–73. [PubMed: 12566312]
40. Sato N, Maitra A, Fukushima N, et al. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer Res* 2003;63:4158–66. [PubMed: 12874021]
41. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005;5:615–25. [PubMed: 16034368]
42. Rhodes DR, Yu J, Shanker K, et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 2004;6:1–6. [PubMed: 15068665]
43. Hudolin T, Juretic A, Spagnoli GC, et al. Immunohistochemical expression of tumor antigens MAGE-A1, MAGE-A3/4, and NY-ESO-1 in cancerous and benign prostatic tissue. *Prostate* 2006;66:13–8. [PubMed: 16114059]
44. Nelson WG, Yegnasubramanian S, Agoston AT, et al. Abnormal DNA methylation, epigenetics, and prostate cancer. *Front Biosci* 2007;12:4254–66. [PubMed: 17485372]

45. Kwabi-Addo B, Chung W, Shen L, et al. Age-related DNA methylation changes in normal human prostate tissues. *Clin Cancer Res* 2007;13:3796–802. [PubMed: 17606710]
46. Bjornsson HT, Sigurdsson MI, Fallin MD, et al. Intra-individual change over time in DNA methylation with familial clustering. *Jama* 2008;299:2877–83. [PubMed: 18577732]
47. Cher ML, Bova GS, Moore DH, et al. Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Res* 1996;56:3091–102. [PubMed: 8674067]
48. Sun J, Liu W, Adams TS, et al. DNA copy number alterations in prostate cancers: a combined analysis of published CGH studies. *Prostate* 2007;67:692–700. [PubMed: 17342750]
49. Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. *Science* 2003;300:489–92. [PubMed: 12702876]
50. Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen YT. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunological reviews* 2002;188:22–32. [PubMed: 12445278]
51. Jager E, Gnjjatic S, Nagata Y, et al. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. *Proc Natl Acad Sci U S A* 2000;97:12198–203. [PubMed: 11027314]
52. Marchand M, van Baren N, Weynants P, et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer* 1999;80:219–30. [PubMed: 9935203]
53. Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* 2008;358:2698–703. [PubMed: 18565862]
54. Small EJ, Sacks N, Nemunaitis J, et al. Granulocyte macrophage colony-stimulating factor--secreting allogeneic cellular immunotherapy for hormone-refractory prostate cancer. *Clin Cancer Res* 2007;13:3883–91. [PubMed: 17606721]

Figure 1.

Overall genomic 5mC content is significantly reduced in metastatic but not primary prostate cancer. **A**, 5mC content is reduced in multiple prostate cancer cell lines compared to normal prostate epithelial and stromal cells. An LC-MS/MS analysis was used to determine the 5mC content as a fraction of total cytosine content in 6 prostate cancer cell lines, normal prostate epithelial cells (PrEC) in primary culture, prostate stromal cells in culture (4ST), WBC DNA pooled from multiple normal male volunteers, and from control specimens. Controls included the M.Oligo which was synthesized with 80% 5mC content, and WBC genomic DNA in which all CpG dinucleotides were methylated to completion with the M.SssI methyltransferase *in vitro* (WBC-SssI). All prostate cancer cell lines, except DU-145, showed reduced 5mC content compared to normal cells. **B**, 5mC content is significantly reduced in metastatic but not primary prostate cancer compared to benign prostate. Application of the same LC-MS/MS approach as above for determination of genomic 5mC content in a series of normal prostate tissues from organ donors (green), tumor adjacent benign prostate tissues from radical prostatectomy specimens, including 11 of 12 containing PIA and PIN prostate cancer precursor lesions (orange), primary prostate cancers from radical prostatectomy specimens (red), hormone naïve lymph node metastases (brown), and metastatic prostate cancers from a rapid autopsy series of men dying of advanced hormone-refractory prostate cancer (maroon). Shown are box-and-whisker plots of the 5mC contents in each specimen. The number of specimens analyzed in each prostate tissue category is indicated.[‡] For the autopsy metastases, 86 metastatic deposits from 28 subjects (1 - 7 mets from each subject) were analyzed. The results of t-test for all

statistically significant differences in the mean $^{5\text{me}}\text{C}$ content between all pairwise combinations are shown. Differences that remained significant after adjusting for age are indicated with *, $p < 0.05$, or †, $p < 0.0005$. *C*, Representative images of immunohistochemistry experiments with anti- $^{5\text{me}}\text{C}$ antibodies in benign prostate sections from organ donors, primary prostate cancer sections from radical prostatectomies, and metastatic prostate cancer sections from a rapid autopsy series, all assembled in tissue microarrays. Each representative section was imaged in both 20x and 40x original magnification as indicated. *D*, Quantitative image analysis of $^{5\text{me}}\text{C}$ immunohistochemistry experiments on prostate tissue TMAs confirms that metastatic prostate cancer tissues (brown), but not primary prostate cancer tissues (red), have significantly reduced genomic $^{5\text{me}}\text{C}$ content compared to normal prostate tissues (green). Shown are box-and-whisker plots of the median intensity as determined by quantitative image analysis of $^{5\text{me}}\text{C}$ stained TMAs. The number of specimens analyzed for each prostate tissue category is indicated. The results of t-test for differences in the mean $^{5\text{me}}\text{C}$ content between all pairwise combinations are shown for all $p < 0.1$. ‡, For the autopsy metastases, 86 metastatic deposits from 28 subjects (1 - 7 mets from each subject) were analyzed. §, For the autopsy metastases, 116 metastatic deposits from 32 subjects (1 - 7 mets from each subject) were analyzed.

Figure 2.

Compared to benign prostate, reduction of LINE1 promoter methylation occurs to some extent in primary prostate cancer, but to a greater extent in metastatic prostate cancer. **A**, An overview of the COMPARE assay used to detect LINE1 hypomethylation as well as gene-specific CpG island hypomethylation. Each sample is split into an untreated fraction and an M.SssI treated fraction in which all CpG dinucleotides are methylated to completion using the M.SssI methyltransferase enzyme. Each fraction is fragmented with restriction enzymes and then densely methylated fragments are captured and enriched with magnetic-bead immobilized MBD2-MBD polypeptides that specifically bind to densely methylated genomic DNA fragments with high avidity. Enriched fragments are eluted and the amount of a given genomic region of interest is determined by real-time PCR. The ratio of the amount of methylated fragments in the untreated fraction to that in the M.SssI treated sample, called the Methylation Index (MI), gives an estimate of the fraction of input genomic DNA fragments that were methylated at a given region of interest. **B**, LINE1 promoter methylation is reduced to varying extents in prostate cancer cell lines compared to normal prostate epithelial (PrEC) and stromal (4ST) cells and normal WBCs as seen by application of the COMPARE assay for detection of methylation at consensus LINE1 promoter regions. **C**, Application of the COMPARE LINE1 promoter hypomethylation assays to genomic DNA from prostate tissues shows that primary prostate cancers (red) and metastatic prostate cancers (brown and maroon) have significantly

reduced LINE1 promoter methylation compared to organ donor normal prostates (green) and tumor adjacent benign prostate tissues including PIA and PIN lesions (green). Shown are box-and-whisker plots of the Methylation Index at LINE1 promoter elements. The extent of LINE1 promoter methylation was even more pronounced in metastatic prostate cancers compared to primary prostate cancers. Number of specimens analyzed for each prostate tissue category is indicated. [‡], For the autopsy metastases, 76 metastatic deposits from 28 subjects (1 - 7 mets from each subject) were analyzed. The results of t-test for all statistically significant differences in the mean ^{5me}C content between all pairwise combinations are shown. Differences that remained significant after adjusting for age are indicated with *, $p < 0.05$, or [†], $p < 0.005$.

Figure 3.

Identification of genes that are not expressed in normal prostate epithelial and stromal cells, but expressed with high confidence in prostate cancer cell lines. Hierarchical clustering and “heat-mapping” of Z-scores from “Bar Code” analysis of gene expression data from Affymetrix hgU133a microarray experiments shows that this analysis identified 168 genes with absence of significant expression in the normal prostate epithelial and stromal cells, but very high confidence of absolute expression in at least one of the prostate cancer cell lines. These genes were considered candidate hypomethylated genes. Green bars indicate known cancer-testis antigen genes identified by this analysis. Red bars indicate genes that were selected for bisulfite sequencing analysis. Details of all genes in this figure are given in Supp. Table S1.

Figure 4.

Hypomethylation of gene specific CpG islands (CpG islands) is correlated with expression of the corresponding genes. *Right Panels*, Extent of methylation at genes that were found to be hypomethylated in prostate cancer cell lines by bisulfite sequencing. For each sample, multiple alleles (number shown to the right of each sample) of each CpG island were cloned and sequenced after bisulfite modification and amplification. The frequency of methylation across all alleles for each CpG dinucleotide was color scaled from black (all alleles methylated) to light blue (none of the alleles methylated). A schematic of each CpG island, with position, relative to the transcriptional start site, of the start and end of the bisulfite sequencing amplicon and the position of each CpG (vertical hash marks) within each amplicon is shown for each

gene above the summary alleles for each specimen. These figures represent a summary of the complete data shown in Supplementary Figure S2, which shows the pattern of methylation in each bisulfite sequenced clone individually. NA, not amplifiable. *Middle Panels*, Extent of methylation at the same genes as in the *Right Panels* as determined by the COMPARE assay. For each sample, the Methylation Index is shown on the x-axis. NA, the M.SssI treated fraction for these specimens were not amplifiable and therefore these specimens were not informative. Data from COMPARE assays closely parallel data from bisulfite sequencing experiments. *Left Panels*, Extent of expression at the same genes as in the *Middle* and *Right Panels* as determined by Affymetrix hgU133A gene expression microarrays. For each sample, the extent of methylation, in normalized arbitrary expression units, is shown on the x-axis. When multiple genes contained identical 5' CpG island sequences, expression levels of all associated genes are shown.

Figure 5.

Gene specific CpG island hypomethylation occurs in primary prostate cancer, but is more pronounced in metastatic prostate cancer. **A**, Box-and-whisker plots showing the Methylation Index as determined by COMPARE assay at each of the CpG islands found to be hypomethylated in the prostate cancer cell lines. The results of t-test for all statistically significant differences in the mean ⁵meC content between all pairwise combinations within each gene are shown, with *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. Statistically significant trends in CTA gene methylation with primary > hormone naïve lymph node metastases > hormone refractory autopsy metastases are indicated with †, $p < 0.05$ or ††, $p < 0.005$. †, indicates that differences remained statistically significant after age adjusting. . The number of specimens analyzed in each prostate tissue category is indicated in the legend. ††, For the autopsy metastases, 76 metastatic deposits from 28 subjects (1 - 7 mets from each subject) were analyzed. **B**, Immunohistochemistry with anti-NY-ESO-1 antibodies in prostate cancer cell lines. NY-ESO-1 protein (encoded by the CTAG1B gene) staining was seen in cell lines that had a high degree of hypomethylation at this gene CpG island (CWR22Rv1 and LAPC-4) but not in the other prostate cancer cell lines. **C**, Immunohistochemistry with anti-NY-ESO-1 antibodies in prostate tissues and control normal testis tissues. No NY-ESO-1 protein staining was observed in any of the benign prostate and primary prostate tissues tested. Robust staining was seen in the testis and in 13% of metastatic prostate cancers. Representative positive staining sections of metastatic prostate tissues and normal testis are shown. Boxed regions are shown in higher magnification. Original optical magnifications are indicated.

Figure 6.

DNA hypomethylation changes are more heterogeneous across different metastatic deposits within the same individual than CpG island hypermethylation changes. **A**, Comparison of the pattern of CpG island hypermethylation changes with the pattern of gene-specific CpG island hypomethylation, LINE1 hypomethylation, and 5meC content reduction changes in multiple metastatic deposits from different subjects. CpG island hypermethylation data, collected by real-time Methylation Specific PCR (RT-MSP), were obtained from a previous study (18) and are color-scaled with red representing complete methylation of >99% (hypermethylation) of input alleles and white representing no methylation. *, in the hypermethylation data indicate that there was a low but detectable level of hypermethylation. DNA hypomethylation data at gene specific CpG islands and at LINE1 promoters, as determined in this study on the same samples and cases as the previous study examining CpG island hypermethylation changes, were color-scaled with white representing >99% of all input alleles being methylated and dark blue representing no methylation (hypomethylation). 5meC content was color scaled with white representing a 5meC content > 0.04 (corresponding to the levels seen in normal WBCs) and dark blue representing no 5meC content. On average, hypomethylation changes were more variable across sites within the same subject compared to the CpG island hypermethylation changes (for gene-specific CpG islands, $p < 10^{-10}$; for LINE1 promoter hypomethylation, $p < 10^{-3}$; for 5meC content, $p < 10^{-5}$). Each subject or case is shown as a distinct module with all of the metastatic sites and methylation data for that subject separated from other subjects. **B**, Representative images of 5meC immunohistochemistry in multiple metastases each from different subjects. The degree of 5meC staining in different metastatic deposits within each

subject was highly variable. Shown are multiple deposits from two representative subjects. The original optical magnification is indicated. *C*, Representative images of NY-ESO-1 immunohistochemistry in multiple metastases each from different subjects. The degree of NY-ESO-1 staining was highly heterogeneous in different metastatic deposits within each subject. Shown are multiple deposits from two representative subjects. The original optical magnification is indicated. *D*, A model of DNA methylation changes in prostate cancer initiation and disease progression.