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Analysis of Promoter Non-CG Methylation in Prostate Cancer

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Abstract

In vertebrates DNA methylation occurs primarily at CG dinucleotides, but recently non-CG methylation has been found at appreciable levels in embryonic stem cells. To assess non-CG methylation in cancer, we compared the extent of non-CG methylation at several biologically important CpG islands in prostate cancer and normal cell lines. An assessment of the promoter CpG islands *Even-skipped homeobox 1 (EVX1)* and *filamin A-interacting protein 1-like (FILIPIL)* demonstrates a 4-fold higher rate of non-CG methylation at *EVX1* compared to *FILIPIL* across all cell lines. These loci are densely methylated at CG sites in cancer. No significant difference in non-CG methylation was demonstrated between cancer and normal. Treatment of cancer cell lines with 5-azacytidine significantly reduced methylation within *EVX1* at CG and CC sites preferentially. We conclude that non-CG methylation does not correlate with CG methylation at hypermethylated promoter regions in cancer. Furthermore, global inhibition of DNA methyltransferases does not affect all methylated cytosines uniformly.

Keywords

non-CpG methylation; epigenetics; prostate cancer; *EVX1*; 5-azacytidine

Introduction

Methylation of cytosines at CG dinucleotides is an important mechanism for genome organization and gene regulation [1]. CG methylation within promoter and intragenic islands plays a role in cancer development and have been widely studied [2]. Recent interest has arisen regarding non-CG methylation, a 5' modification that occurs at cytosines adjacent to non-CG dinucleotides (CA, CT, or CC). This process occurs extensively in plants *de novo* and is mediated by distinct methyltransferases *Chromomethylase 3*, *DRM1*, and *DRM2* [3]. Non-CG methylation in vertebrates is found in human embryonic stem cells (ESC) and embryos with a near absence in somatic cells [4–6]. Non-CG methylation in stem cells may amount to almost 25% of all methylated cytosines, in contrast to an average of over 90% at CG sites. Vertebrates contain several methyltransferases including DNMT1 involved in maintenance methylation and DNMT3a and b that have *de novo* functions. In mammalian cells, the methyltransferase DNMT3a has been implicated as a possible mediator of non-CG methylation with non-CG methylation correlating with DNMT3 expression levels [4, 7]. These data suggest that loss of non-CG methylation occurs during differentiation and that non-CG methylation may be a unique feature to mammalian stem cell populations.

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Cancer cells exhibit many features associated with stem cells including self-renewal [8]. Evidence has now begun to emerge that non-CG methylation exists in human cancer cells. Early work had demonstrated non-CG methylation at the *p16* locus in breast cancer [9]. However, recent genome-wide approaches suggest non-CG methylation is a relatively rare epigenetic phenomenon that occurs at specific loci [10]. Functionally, an early paper had reported that non-CG methylation may alter protein binding to the B29 gene promoter in cancer altering transcription although whether this is a wider phenomenon is debated [11]. Factors that regulate non-CG methylation in cancer cells are unknown.

The relative scarcity of research on this topic in mammals is related to the technical challenges in methylation analysis. Use of high-throughput methylation-specific PCR based technologies is impractical given the infrequent occurrence of non-CG sites in mammalian cells [3]. Furthermore, non-CG methylation occurs at very specific loci during development [12], and discovery and identification of such loci in adult stem cell populations requires genome-wide analysis. Currently bisulfite sequence is the predominant method for detection of non-CG methylation. Yan *et al.* reported that non-CG methylation is reduced after subsequent rounds of PCR, suggesting the decreased affinity of bisulfite sequencing primers to sequences containing non-CG methylation results in a dramatic underestimation of non-CG methylation rates reported in previous studies [13]. Due to the challenges associated with current methods of detection, the analysis of non-CG methylation remains underdeveloped.

In this study, we compared the presence of non-CG methylation in prostate cancer cells (PCa) and human prostate epithelial cells at several known CpG islands, *even-skipped homeobox 1 (EVX1)* and *filamin A-interacting protein 1-like (FILIPIL)* that demonstrate hypermethylation in cancer and are functionally important [14] [15]. We find that non-CpG levels are higher in cancer compared to non-cancer cells. The DNA methyltransferase inhibitor 5-azacytidine (AzaC) significantly decreases CG and CC methylation levels preferentially. These results support a role for non-CpG methylation in cancer, and suggest that CA and CT methylation may not be dependent on DNA methyltransferases that are inhibited by 5-azacytidine.

Materials and Methods

Cell Culture, DNA extraction, and Bisulfite Conversion

PPC-1 and PC3 cells were purchased from the American Type Culture Collection and cultured in DMEM (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum, and 1% penicillin-streptomycin. PC3 cell line was treated with 2.5 μ M of the demethylating agent 5-azacytidine (AzaC) (Sigma-Aldrich, St. Louis, MO) for 48h. Cells were redosed with AzaC after the first 24 hours of treatment. Normal HPEC were established on collagen-coated dishes in Ham's F-12 supplemented medium containing 1% fetal bovine serum as previously described [16]. DNA was isolated from HPEC and PCa cell lines using DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). Sodium bisulfite conversion was performed using Epitect Bisulfite Kit (QIAGEN). HPEC DNA samples from several individual patients were collected. This study was approved by the Institutional Review Board (IRB) at the University of Wisconsin.

Bisulfite Sequencing

Three sets of primers spanning 25–1484 downstream of the *EVX1* transcriptional start site (TSS) were used to amplify the first two CG islands [14]. Bisulfite sequencing was also performed across 180 b.p. at the *FILIPIL* locus as previously described [15]. Amplification of *EVX1* and *FILIPIL* were performed using the same bisulfite-converted DNA samples.

PCR was performed to amplify bisulfite-converted DNA using HotStarTaq according to the manufacturer's recommendations (QIAGEN). The following cycling conditions were used: 95° C for 15 min; 50 cycles of 95° C for 15 sec, 55° C for 30 sec, 72° for 90 sec. PCR products were cloned into pCR2.1 using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). We collected 10–20 colonies for each region assessed. Plasmid isolation was performed using PureYield Plasmid Miniprep System (PROMEGA, Fitchburg, WI) kit and sequenced. A total of 5–10 sequencing results per region per cell type were obtained and aligned using BiQ Analyzer [17]. Bisulfite sequencing regions for *EVX1* and *FILIP1L* are diagrammed in Figure 1A. Occurrences of cytosines in the non-CG context after bisulfite sequencing will be abbreviated as “CH” according to the convention established by previous studies [4].

Statistical Analysis

Microsoft Excel scripts were generated to align sequences, calculate 30 base-pair window methylation densities, and quantify the frequency of individual dinucleotides. The frequencies of CT, CC, CA, and CG dinucleotides were compared between cell lines using the two-tailed Fisher's exact test. The average % methylation of CG and CH dinucleotides were compared using the Mann-Whitney and t-tests. All statistical analyses were performed using SPSS Version 10.0 (IBM). All statistical tests were two-tailed. A *P* value of < 0.05 was considered statistically significant.

Results

Areas of methylation that clearly regulate gene transcription are CG-enriched and placed typically within the promoter and gene body. We have previously described *FILIP1L* and *EVX1* as some of the most frequently hypermethylated genes in PCa [14, 15]. In the present study, we assessed non-CG methylation (CC, CT, CA) at these two loci in PC3 and PPC-1 prostate cancer cell lines and human prostate epithelial cells (HPECs) using bisulfite sequencing. Gene structure and CG density across the gene promoters are shown (Figure 1A). Within the 1200 bp region of *EVX1* there were 474 cytosines in sum with 21% CG and 79% non-CG. *FILIP1L* contained 154 cytosines with 37% CG and 63% non-CG. To control for variations in bisulfite conversion, loci were analyzed on the same DNA samples.

In the *FILIP1L* CpG island, non-CG methylation was found to be infrequent in all cell lines assessed, accounting for only 1.5% of non-CG cytosines. In contrast, approximately 4% of all non-CG cytosines were methylated at the *EVX1* locus (Figure 1B). When compared to *FILIP1L*, this difference was statistically significant ($P < 0.0001$) suggesting that non-CG methylation is locus specific. We then compared CG methylation at both *EVX1* and *FILIP1L*. PC3 and PPC-1 were densely hypermethylated across these CpG islands when compared to non-cancer HPECs ($P < 0.0001$). In contrast, no difference in non-CG methylation was observed when PCa cell lines were compared to non-tumor cells (Figure 1B).

We then regionally compared CG to non-CG methylation across *EVX1* using a 30-bp window (Fig. 2). This analysis was not performed for *FILIP1L* given the low levels of non-CG methylation. In PC3 and PPC1, CG and non-CG methylation are widely distributed across *EVX1*. In HPECs, CG methylation was consistently decreased in the upstream regions of all three normal cell lines. In summary, there was not a spatial relationship between non-CG and CG methylation in cancer and normal cell lines.

The methylation inhibitor 5-azacytidine (AzaC) forms a reaction intermediate 5-aza-2'-deoxycytidine which binds covalently to DNA and results in pan-DNMT inhibition and degradation [18]. To test the role of inhibiting methyltransferases on non-CG methylation in cancer cells, we treated PC3 with 2.5uM of AzaC for 48h prior to assessing DNA

methylation. This dose has been demonstrated previously to induce *EVX1* gene expression [14]. AzaC treatment significantly reduced both CG (86% to 36%) and non-CG methylation (1.2% to 0.19%) ($P < 0.0001$ and $P < 0.0001$, respectively) (Figure 3A). Treatment with AzaC preferentially decreased the frequency of CG and CC dinucleotides compared to CA and CT ($P < 0.0001$, and $P = 0.03$, respectively) (Figure 3B). A reduction from 1.2% to 0.2% was observed for methylation at CC. Analysis of CG methylation density reveals that AzaC treatment demethylates CG across several areas of the *EVX1* gene including upstream sequences (Figure 3C). We conclude that AzaC inhibits CG methylation, but also decreases CC methylation preferentially.

Discussion

Non-CG methylation has engendered increased interest recently with the recognition of enrichment in stem cells and the development of genome-wide analyses. Our study is one of the first to compare non-CG methylation at CpG islands in cancer and normal epithelial cells. The CpG islands we evaluated have been shown previously to be densely methylated at CG dinucleotides in cancer [1]. In contrast, we find that non-CG methylation does not differ between cancer and normal. In addition, we find that in cancer cells, inhibition of DNA methyltransferases using a global inhibitor results in a preferential loss of CG and CC methylation.

Previous studies have reported the more frequent occurrence of non-CG methylation in stem cells compared to more differentiated cells [4–6], although others have found wide variation between pluripotent cell lines [6]. Many cancers contain a stem cell phenotype leading us to speculate differences exist between cancer and normal. We utilized several known loci that demonstrate dense CG methylation in cancer cells and are functionally important [14, 15]. We did not find a significant difference in non-CG methylation between cancer and non-cancer cells at these loci. Others have reported a close association between CG methylation and non-CG methylation on a genome-wide scale in stem cells [4, 6]. Analysis of *FILIP1L* did not demonstrate the unusually high occurrence of non-CG methylation observed at the *EVX1* locus, suggesting that non-CG methylation is locus specific and not always correlated with CG methylation in cancer. Our data also suggests that non-CG methylation and CG methylation are not mutually exclusive consistent with earlier reports [11, 20].

Previously, Ziller *et al.* demonstrated that knockdown of *DNMT3a* resulted in a global reduction in non-CG methylation [6]. Our study is the first study to demonstrate a decrease in non-CG methylation using a well-known DNA methyltransferase inhibitor. Treatment of PC3 cells with 5-azacytidine resulted in a significant 2-fold reduction in CG methylation and a 3-fold reduction in non-CG methylation ($P < 0.0001$, Figure 2A). We further find that 5-azacytidine preferentially reverses only CG and CC methylation, while leaving CA and CT methylation intact. The implication of this finding is not clear but suggests CA and CT methylation may not be dependent on DNA methyltransferases that are inhibited by 5-azacytidine.

Non-CG methylation has been an area of controversy due to the possibility of incomplete bisulfite conversion [13]. However, there is increasing evidence for the existence of non-CG methylation in mammals with the development of genome-wide techniques for analysis [6, 13]. In our study, analysis of a second locus, *FILIP1L*, revealed much lower frequencies of non-CG methylation compared to *EVX1*, a pattern consistent across both normal and cancerous cell lines. If the high frequency of non-CG methylation at *EVX1* were due to incomplete conversion, then similar rates of non-conversion would have been observed across different genomic regions. One limitation of our study is that non-CpG methylation was evaluated across only two loci. However, these are biologically significant regions in

cancer and demonstrate clear differences in non-CG methylation. Previous reports have found elevated non-CG methylation at functionally important promoters [21, 22].

The role of non-CG methylation in mammalian cells remains unclear. It would be expected that if non-CG methylation were maintained at a high fidelity a depletion of these sites were occur through deamination; a process that does not occur. In plants, non-CG methylation plays a role in gene silencing and genomic imprinting [3]. Whether this occurs in cancer is unknown. Our finding of localized high levels of non-CG methylation at *EVX1*, and decreased non-CG methylation with inhibition, might suggest a role in expression inhibition. *EVX1* is an important gene during development, a time when CG methylation levels are reduced [23]. Alternately, low levels of non-CG methylation are seen at a locus, *FILIP1L*, that in a similar fashion to *EVX1* undergoes reexpression with AzaC exposure. Further studies are required to fully elucidate the functional significance of non-CG methylation within promoter regions in cancer.

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Abbreviations

AzaC	5-azacytidine
CH	non-CG methylation
CSC	cancer stem cells
ESC	embryonic stem cells
EVX1	even-skipped homeobox 1
HPEC	human prostate epithelial cells
PCa	prostate cancer

References

* = of interest

** = of considerable interest

1. Law JA, Jacobsen SE. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet.* 2010; 11(3):204–220. [PubMed: 20142834]
2. Shenker N, Flanagan JM. Intragenic DNA methylation: implications of this epigenetic mechanism for cancer research. *Br J Cancer.* 2012; 106(2):248–253. [PubMed: 22166804]
3. Dyachenko OV, Schevchuk TV, Kretzner L, Buryanov YI, Smith SS. Human non-CG methylation: are human stem cells plant-like? *Epigenetics.* 2010; 5(7):569–572. [PubMed: 20647766]
4. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature.* 2009; 462(7271):315–322. [PubMed: 19829295]
5. Laurent L, Wong E, Li G, et al. Dynamic changes in the human methylome during differentiation. *Genome Res.* 2010; 20(3):320–331. [PubMed: 20133333]
6. Ziller MJ, Müller F, Liao J, et al. Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. *PLoS Genet.* 2011; 7(12):e1002389. [PubMed: 22174693]
7. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc Natl Acad Sci USA.* 2000; 97(10):5237–5242. [PubMed: 10805783]

8. Gupta PB, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? *Nat Med.* 2009; 15(9): 1010–1012. [PubMed: 19734877]
9. Woodcock DM, Linsenmeyer ME, Doherty JP, Warren WD. DNA methylation in the promoter region of the p16 (CDKN2/MTS-1/INK4A) gene in human breast tumours. *Br J Cancer.* 1999; 79(2):251–256. [PubMed: 9888465]
10. Pulverer W, Wielscher M, Panzer-Grümayer R, et al. The stem cell signature of CHH/CHG methylation is not present in 271 cancer associated 5'UTR gene regions. *Biochimie.* 2012
11. Malone CS, Miner MD, Doerr JR, et al. CmC(A/T)GG DNA methylation in mature B cell lymphoma gene silencing. *Proc Natl Acad Sci USA.* 2001; 98(18):10404–10409. [PubMed: 11504918]
12. Haines TR, Rodenhiser DI, Ainsworth PJ. Allele-specific non-CpG methylation of the Nf1 gene during early mouse development. *Dev Biol.* 2001; 240(2):585–598. [PubMed: 11784085]
13. Yan J, Zierath JR, Barrés R. Evidence for non-CpG methylation in mammals. *Exp Cell Res.* 2011; 317(18):2555–2561. [PubMed: 21925168]
14. Truong M, Yang B, Wagner J, et al. Even-skipped homeobox 1 is frequently hypermethylated in prostate cancer and predicts PSA recurrence. *Br J Cancer.* 2012
15. Desotelle JT, Matthew, Ewald Jonathan, Weeratunga Pushpa, Yang Bing, Huang Wei, Jarrard David F. CpG Island Hypermethylation Frequently Silences FILIP1L Isoform 2 Expression in Prostate Cancer. *Journal of Urology.* 2012
16. Schwarze SR, Shi Y, Fu VX, Watson PA, Jarrard DF. Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells. *Oncogene.* 2001; 20(57):8184–8192. [PubMed: 11781834]
17. Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics.* 2005; 21(21): 4067–4068. [PubMed: 16141249]
18. Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. *J Natl Cancer Inst.* 2005; 97(20):1498–1506. [PubMed: 16234563]
19. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene.* 2002; 21(35):5427–5440. [PubMed: 12154405]
20. Franchina M, Kay PH. Evidence that cytosine residues within 5'-CCTGG-3' pentanucleotides can be methylated in human DNA independently of the methylating system that modifies 5'-CG-3' dinucleotides. *DNA Cell Biol.* 2000; 19(9):521–526. [PubMed: 11034545]
21. Kouidou S, Agidou T, Kyrkou A, et al. Non-CpG cytosine methylation of p53 exon 5 in non-small cell lung carcinoma. *Lung Cancer.* 2005; 50(3):299–307. [PubMed: 16125822]
22. Nishino K, Hattori N, Sato S, et al. Non-CpG methylation occurs in the regulatory region of the Sry gene. *J Reprod Dev.* 2011; 57(5):586–593. [PubMed: 21636956]
23. Briata P, Van De Werken R, Airolidi I, et al. Transcriptional repression by the human homeobox protein EVX1 in transfected mammalian cells. *The Journal of biological chemistry.* 1995; 270(46): 27695–27701. [PubMed: 7499236]

Executive Summary

Non-CG methylation is a well-established phenomenon in plants, but studies in mammalian cells especially cancer are rare..

- Recently, genome-wide methylation analysis demonstrated a high occurrence of non-CG methylation in human embryonic stem cells, while a near absence was observed in somatic cells.

Even-skipped homeobox 1 (EVX1) was previously found to be an important locus in which gene expression was epigenetically controlled in human prostate cancer.

- Prostate cancer tissue specimens demonstrated high CG methylation compared to normal specimens.
- EVX1 hypermethylation predicts PSA recurrence after prostatectomy.

A high occurrence of non-CG methylation was found at the EVX1 locus.

- Non-CG methylation was analyzed at 2 loci *EVX1* and *filamin A-interacting protein 1-like (FILIP1L)*.
- A 4-fold greater frequency of non-CG methylation was observed at *EVX1* compared to *FILIP1L*.

5-azacytidine, a DNA methyltransferase inhibitor, decreases non-CG methylation 3-fold in PC3 cells

- Previous studies have implicated a role for DNA methyltransferases in maintaining non-CG methylation.
- This study shows that non-CG methylation is reversible with the demethylating agent 5-azacytidine.

Conclusions

- A growing number of studies are reporting non-CG methylation in a variety of mammalian cell lines.

DNA methyltransferases may be responsible for maintaining aberrant non-CG methylation in prostate cancer.

Figure 1.

A. Diagram of *even-skipped homeobox 1* (*EVX1*) and *filamin A-interacting protein 1-like* (*FILIP1L*). Bisulfite sequencing was performed encompassing the promoter CpG islands of *EVX1* and *FILIP1L* Isoform 2. CG density (tic marks) are displayed for each locus. **B.** Frequency of methylated cytosines at the *EVX1* and *FILIP1L* loci. The frequency of methylated cytosine followed by T, C or A (left) or G (right) was calculated at each locus in cancer cell lines and 3 normal human prostate epithelial cell (HPEC) lines. A significantly higher rate of non-CG methylation (4%) was observed at *EVX1* compared to *FILIP1L* ($P < 0.0001$) where non-CG methylation is a rare event (1.5%). Increased CG methylation was observed in PCa cell lines compared to HPECs ($P < 0.0001$), but non-CG methylation was not. Each cell line represents an average of 4-6 clones. The same bisulfite-converted DNA samples were used to amplify both *FILIP1L* and *EVX1* to avoid variability related to incomplete bisulfite conversion.

Figure 2. Regional methylation density at CG and non-CG sites across the EVX1 locus
Methylcytosine density within 30-bp windows was calculated at each base pair and plotted on the y-axis. In HPECs, regions surrounding the transcription start site were unmethylated at CG compared to cancer cell lines. No correlation between CG and non-CG methylation was observed.

Figure 3. Effect of 5-Azacytidine (AzaC) on CG and non-CG methylation

PC3 prostate cancer cells were treated with AzaC or DMSO for 48 h before DNA extraction, bisulfite sequencing and cloning. **A.** AzaC significantly reduced CG and CH methylation at the *EVX1* locus. Error bars represent 95% confidence intervals. **B.** AzaC treatment of PC3 decreased the frequency of non-CG methylation and preferentially reduced levels of methylated CC dinucleotides ($P = 0.03$). **C.** AzaC treatment reduces 30-b.p. window methylation densities at CG across *EVX1* near the transcription start site. No regional changes in non-CG methylation are noted.