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Global methylation pattern of genes in androgen sensitive and androgen independent prostate cancer cells

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Abstract

Promoter DNA methylation of CpG islands is an important epigenetic mechanism in cancer development. We have characterized the promoter methylation profile of 82 genes in three prostate cancer cell lines (LNCaP, PC3 and DU145) and two normal prostate cell lines (RWPE1 and RWPE2). The methylation pattern was analyzed using a Panomics gene array system that consists of immobilized probes of known gene promoters on a nitrocellulose membrane. MBP (methylated binding protein) purified methylated DNA was hybridized on the membrane and detected by the chemiluminescence method. We analyzed methylation profile in normal (RWPE1) vs cancerous cells and AR-sensitive (LNCaP) vs AR-negative cells (DU145 and PC-3). Our study demonstrates that more than 50% of the genes were hypermethylated in prostate cancer cells as compared to 13 % in normal cells lines. Among these were the tumor suppressor (Rb, TMS-1, DAPK, RBL1, PAX-6, FHIT); cell cycle (p27KIP1, CDKN2A); transporters (MDR1, MLC1, IGRP); and transcription factor (STAT1, CIITA MYOD, NPAT) genes. Relative methylation pattern shows that most of these genes were methylated from 5 to >10-fold as compared to the normal prostate cells. In addition, promoter methylation was detected for the first time in target genes such as RIOK3, STAT5, CASP8, SRBC, GAGE1 and NPAT. A significant difference in methylation pattern was observed between AR-sensitive vs AR-negative cancer cells for the following genes: CASP8, GPC3, CD14, MGMT, IGRP, MDR1, CDKN2A, GATA3 and IFN genes. In summary, our study identified candidate genes that are methylated in prostate cancer.

Keywords

DNA Methylation; prostate cancer; promoter gene Array

Introduction

Epigenetic mechanism is a vital event in the transcriptional regulation of various genes in eukaryotes. It includes aberrant DNA methylation and histone modification at multiple levels and play important role in normal developmental processes, gene imprinting and human

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carcinogenesis (1). Aberrant DNA methylation of CpG (Cytosine preceding Guanosine) sites is among the earliest and most frequent alterations in cancer (2,3). This modification has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands, located in the promoter regions of many genes. In many cases, aberrant methylation of the CpG island in genes has been correlated with a loss of gene expression and function (4,5). Markers for aberrant methylation may represent a promising avenue for monitoring the onset and progression of cancer. Identification of promoter methylation of several genes in small biopsies and bodily fluids of cancer patients has proven to be useful as a molecular tool for cancer detection and progression (6).

Prostate cancer is the second leading cause of cancer death among the men in the USA and Europe (7). This disease is associated with considerable morbidity and mortality, but curative treatment (radical prostatectomy or radiotherapy) is feasible for patients with the early-stage disease (8). However, our understanding of the epigenetic changes that underlie the progression of this disease remains at an early stage. Association studies predicted a number of genetic factors associated with risk of prostate cancer in different populations but there is still scarcity of data on the epigenetic events. A better understanding of the molecular and epigenetic changes in prostate cancer is likely to contribute to improved diagnosis, clinical management, and better treatment outcomes. A recent study has shown that smoking influences aberrant CpG hypermethylation of several genes in prostate cancer (9).

There are several molecular methods like methylation specific PCR (MS-PCR), methylation specific digestion and bisulfite sequencing for methylation analysis. However, they can screen only a few sites of methylation at one time. In our current study we screened the promoter methylation pattern of 82 genes (at one time) using an array method in three prostate cancer cell lines (LNCaP, DU145 and PC3) and in one normal prostate cell line (RWPE1). We investigated promoter methylation patterns in various categories of genes such as cell cycle regulators, transcription factors, tumor suppressor and genes involved in tumor growth and progression. Moreover, an analysis was done to explore the difference in methylation pattern between the androgen sensitive and androgen-independent prostate cancer cells. A distinct methylation pattern was observed between normal and prostate cancer cell lines as well as androgen sensitive and androgen-independent cell lines. This study will provide the opportunity to investigate the potential role and mechanism of novel genes in prostate cancer development and progression.

Materials and Methods

Cell culture

Human prostate normal and cancer cell lines (RWPE1, RWPE2, LNCaP, DU145, PC3) were obtained from American Type Culture Collection (Manassas, VA). RWPE1 and RWPE2 are normal prostatic epithelial cell lines which were isolated from a histologically normal adult human prostate. These cells are androgen receptor (AR), p53 and pRb positive. Monolayer cultures were maintained in epithelial cell enrichment media (Epi-media: DMEM/F12 with 5% Horse Serum; EGF 20ng/ml; Insulin 10μg/ml; Cholera Toxin 100ng/ml; Hydrocortisone 500ng/ml; HEPES 15mM). Prostate cancer cells, LNCaP are androgen sensitive (AR positive) cells derived from a patient with metastatic site to the left supraclavicular lymph node. DU145 and PC3 are androgen independent (AR negative) cells. They secrete PSA (prostate specific antigen) and KLK-2 (Kallikrein-2). PC3 and DU145 cells are derived from a patient with grade IV prostatic adenocarcinoma that had metastasized to the brain and bone respectively. These DU145 and PC3 cells were maintained in DMEM/F12 medium containing 10% fetal bovine serum and 4 mM L-glutamine. Cells were harvested when they reached 80% confluence.

Promoter Methylation Analysis (Panomics)

Genomic DNA from cell lines was isolated by Qiamp DNA mini Kit (Qiagen Inc). Methylation profile study was carried out using Promoter Methylation Array kit (Panomics Inc). Briefly, 2 μg of genomic DNA was digested with 10U MseI (New England Biolab) to produce small fragments of DNA which retain the CpG islands and was purified. These DNA fragments were further ligated with linker DNA for PCR analysis. In the next step, DNA was incubated with methylation binding protein (MBP) in presence of binding buffer at 15°C for 30 minutes which forms a protein/DNA complex. The methylated DNA fragments were isolated by centrifugation using a separation column. Next, the purified methylated fragments were converted into the biotinylated probe by PCR amplification in the presence of biotin-dCTP for 30 cycles at 94°C for 1min, 55°C for 1min and 72°C for 2 min. During PCR, hybridization membranes were pre-treated with pretreatment buffer (provided with the kit) at room temperature and then biotinylated probes were hybridized to the methylation array membrane in presence of hybridization buffer (provided with kit) at 50°C for overnight in a rotating hybridization oven. Next day, the membrane was washed, incubated in blocking buffer for 15 min and then Streptavidin-HRP conjugate was added to the membrane. After washing, the membrane was incubated with 1X detection buffer for 5 min at room temperature and then the substrate was added as provided with the kit. The images were developed with chemifluoresence reagent. Spot intensities on the membrane were determined by using Quantity One Fluor-S Imaging device (BIORAD). The promoter microarray contains duplicate spots of 82 gene promoter sequences, 28 positive controls and 12 non-promoter controls (Table 1). Microarray data were normalized within-array normalization using positive controls (mean volume optical density of gene of interest/ mean volume optical density of positive controls). A relative methylation fold was obtained in LNCaP, PC3 and DU145 compared to the corresponding gene methylation OD (optical density) in RWPE1 cell line.

Promoter methylation PCR

Promoter methylation PCR was performed to validate the quantitative results obtained from the Panomic array. We randomly selected up to seven genes and performed the promoter methylation PCR. Two μg of genomic DNA was digested with 10U MseI, incubated with methylated binding protein to form a protein/DNA complex (as above). Methylated DNA was further separated through column and amplified with promoter specific primers (Table 2) for CASP8, CD14, HOXA2, MASPIN, MDR1, TMS1 and STAT1 at the following program: 94° C for 5min, 94°C for 1min, 56°C for 1min and 72°C for 2min for 35 cycles.

Methylation specific PCR

Furthermore, to confirm and validate the methylation results from Panomics array, we performed the methylation specific PCR of CASP8, MASPIN, MDR1 and TMS1 genes followed by bisulfite sequencing of TMS1 and MDR1 genes. Bisulfite modification of 2 μg of DNA was done using EZ DNA methylation kit (Zymo Research, USA). Briefly, DNA was mixed with 130μl of CT conversion reagent and kept in PCR machine at 98°C for 10min, 64° C for 2.5hour and finally at 4°C for 1 hour. Next, these samples were added to Zymo-Spin column containing 600μl M-binding buffer. Samples were centrifuged at 13000g for 30 seconds, washed with M-wash buffer. For desulfonation, 200μl M-Desulfonation buffer was added to column and incubated for 20 minutes and again centrifuged at 13000g for 30 seconds. The column was washed twice with washing buffer and then modified DNA was eluted in 10μl of M-elution buffer. The modified DNA was used for methylation specific PCR for CASP8, TMS1, MASPIN and MDR1 and for bisulfite sequencing for TMS1.

RNA expression Analysis

RNA was extracted by Trizol (Invitrogen) method. cDNA was prepared using Thermoscript RT kit (Invitrogen) and 2 μl of each cDNA was used for Real time PCR assay using SYBR green. Gene expression of few genes such as CASP8, TMS1, MDR1 and MASPIN was analyzed before and after the treatment with 5μmol/L of demethylating agent 5-Aza-2'-deoxycytidine for 72 hours.

Data Analysis

We have calculated the Mean ± SD (OD) of methylation fold of each gene in normal prostate and prostate cancer cell lines.

Results

The Panomics promoter methylation array technique enabled us to screen the methylation status of 82 genes in prostate cancer cell lines at one time. This technique was quite simple and sensitive. In this genome-wide global methylation assay, more than 50% of the 82 genes were methylated in prostate cancer cell lines as compared to 13% methylated genes in normal prostate cell line i.e. RWPE1. Androgen independent cell lines, DU145 and PC3 expressed 64.6% and 61% methylated genes respectively as compared to 53.7% methylated genes in androgen sensitive cell line i.e. LNCaP cells (Figure 1). Furthermore, when the methylated genes were sub-grouped according to their function, we found an increased methylation in number of genes belonging to tumor suppressor category followed by transcription factors, cell cycle, angiogenesis, immune related, apoptosis and transporter genes.

Normal prostate cells (RWPE1 and RWPE2) also expressed methylated genes albeit in smaller numbers - 2.4% tumor suppressor and oncogenes, 1.2% immune related and apoptotic genes, and almost 4.9% belonging to other categories. No hypermethylation was observed in cell cycle, angiogenic and transporter genes in normal prostate cells. The Androgen sensitive prostate cancer cell, LNCaP showed a significant increase in hypermethylation of genes that belong to tumor suppressor; transcription factors (9.8%); immune related (6.1%); oncogenes (3.6%); cell cycle (3.6%); angiogenesis (4.9%) and transporter genes (3.7%). AR-negative prostate cancer cells DU145 and PC3 showed similar pattern as the AR-sensitive prostate cancer cells, with the exception of a greater number of hypermethylated genes belonging to the transcription factor (11–12%) and cell cycle (6–7%) categories. These observations suggest that there are selective differences in patterns between normal and cancer cells and androgen sensitive and androgen independent prostate cancer cells.

Furthermore, we compared the mean hypermethylation intensity in Androgen-sensitive and Androgen-independent prostate cancer cells in reference to non-cancer prostate cell RWPE 1 (cut off value 5 fold. The data from RWPE2 normal prostate cells were similar to RWPE1, Hence these data are not shown). Out of 82 genes screened, 58 were hypermethylated to different levels in Androgen-sensitive and Androgen-independent prostate cancer cells. Some of the candidate genes belonging to different functional categories according to their function are listed in Table 3.

Difference in DNA methylation pattern between normal and prostate cancer cells

We observed a considerable difference in the promoter methylation array pattern between normal prostate cells (RWPE1) and prostate cancer cells (LNCaP, DU145 and PC3). Figure 2 (A) represents a heat map of all the 82 genes showing extent of methylation in all cancer cell lines as compared to normal RWPE1. The genes are grouped according to their relative methylation fold: ≥10 fold; 5–10 fold; 2–5 fold; and <2-fold. Although their relative intensity of methylation varied from one cell type to other, they exhibited similar pattern of methylation

except for few genes. Tumor suppressor genes such as TMS-1, RB, RBL1, DAPK, SFN, 14-3-3 sigma, PAX6, FHIT and SRBC were highly methylated (more than 10-fold) while Maspin and DBCCR1 were moderately methylated (5–6 fold). Transcription factors such as STAT5, MYOD, NPAT, ATF2, WT1, HOXA2, SYBL1, STAT1; cell cycle and DNA repair genes such as p27KIP1, TP73, CDKN2A, p21, K-RAS were also high to moderately methylated in prostate cancer cell lines. Genes belonging to transporters (MDR1, MLC1,CFTR, MTX-1); immune system (IFN, KIR2DL4, GAGE1, BAGE, LAGE-1, IL4) and angiogenesis (TIMP3, TSP1, PAI1) were moderately to highly methylated in prostate cancer cells. Methylation in some of these genes such as PAX6, SRBC, ATF2, STAT5, LAGE 1, NPAT, CFTR, MTX-1, IL4 and RIOK3 in prostate cancer was not reported so far.

Difference in methylation pattern between Androgen sensitive and Androgen-independent prostate cancer cells

Increased DNA methylation was observed in AR negative cells (DU145 and PC3) as compared to the AR positive cell (LNCaP). CASP8, GPC3, CD14, MGMT, IFN, GATA-3, H-RAS, SIM2, MDR1, IGRP, WT1, CDKN2A, PDGF-B genes showed more than 2-fold methylation in AR independent cell lines as compared to androgen sensitive cell lines. In contrast, decreased methylation was observed in some tumor suppressor genes (RBL1, RB, TMS1, DAPK), transcription factors (HOXA2, STAT5, STAT1), cell cycle genes (CyclinD2, K-ras) and angiogenic genes (PAI-1, TIMP3) (Figure 2 B).

Methylation Specific PCR and gene expression analysis

Promoter methylation PCR by Panomics kit confirms that genes present on the array are methylated (Figure 3). CASP8, HOXA2 and MDR1 shows the presence of some methylated copies in RWPE1 cells. Our bisulfite methylation specific PCR (MSP) results showed that MASPIN and MDR1 are completely unmethylated while CASP8 and TMS1 are expressed in both methylated and unmethylated forms in normal cell line (RWPE1) (Figure 4A). These sets genes are strongly methylated in cancer cell lines as compared to normal cell line (RWPE1). Bisulfite sequence analysis of TMS1 also suggested that the promoter is unmethylated in RWPE1 cell lines, completely methylated in LNCaP and Du145 and partially methylated in PC3 (Figure 4C and 4D). Bisulfite sequence analysis of MDR1 gene also showed one methylated site in RWPE1, two in DU145 and four methylated sites in PC3 cells. LNCaP cells showed only partially methylated sites. After 48 hr of treatment with AZC several genes became demethylated however the reversal to demethylation was not complete (Figure 4B). Presence of methylated bands suggests that genes were not completely demethylated after 48 hours of treatment.

One of the most obvious outcome from DNA methylation is reduced mRNA expression of that gene. Our RNA expression data support that these genes are methylated in cancer cell lines with a corresponding decrease in their gene expression compared to normal RWPE1 cell line (Figure 5A). Gene expression was also restored for MDR1, TMS1 and MASPIN genes after 5-aza-dC treatment (Figure 5B). Only CASP8 gene showed non-significant restoration in LNCaP and DU145 cell lines (Figure 5B) while in PC3 its expression was reduced.

Effect of inhibiting DNA methylation on MDR1 expression in Androgen sensitive and Androgen-independent prostate cancer cells

We have demonstrated the effect of DNA methylation on target gene MDR1 in AR-independent (PC3, DU145), and AR+ sensitive cell (LNCaP) lines, as well in a normal prostate cell line RWPE1 (figure 6). Our data clearly demonstrate functional differences in re-expression of MDR1 upon inhibition of DNA methylation by AZC and subsequent treatment with docetaxol. RWPE1, non-tumorigenic prostate cells express good levels of constitutive MDR1 protein, as assessed by Immunofluorescent analysis. Pretreatment with AZC, followed

by docetaxol treatment had no significant effect on MDR1 expression. In contrast, AR-negative prostate cancer cells PC3 and DU145, which had significant loss of MDR1 due to its promoter methylation, showed a significant restoration of MDR1 upon AZC treatment. These cells showed further increase in MDR1 expression in response to AZC plus docetaxol treatment. Interestingly, LNCaP, AR+ive or responsive cells, did show some response in restoring MDR1 expression. However, the restoration was not as marked as for PC3 and DU145 (AR-ive cells). These observations suggest that there are functional differences between AR+ and AR− prostate cancer cells in their response to inhibitors of DNA methyaltion and chemotherapeutic agent docetaxol.

Effect of AZC alone or in combination with docetaxol on growth inhibition of normal and cancer prostate cells

Since, several target genes, including MDR1 were silenced due to DNA methylation; we examined the effect of docetaxol on normal and prostate cancer cells, where MDR1 was restored by inhibiting DNA methylation. Supplementary figure 4 shows that docetaxol alone or after treatment with AZC had no effect on growth inhibition of normal prostate cells RWPE1. In addition, docetaxol alone had very little effect on growth inhibition of prostate cancer cells PC3 and DU145. However, when prostate cancer cells were pre-treated with AZC for 24 hrs, there was a significant increase in docetaxol induced growth inhibition of both prostate cancer cells.

Discussion

In this study, we investigated the differential DNA methylation pattern between cancer vs non-cancer and androgen-sensitive vs androgen-independent prostate cancer cells by a TranSignal promoter methylation array technology (Panomics Inc.). This technique provides a high throughput analysis of promoter methylation of 82 genes simultaneously. The principle behind this technique is the isolation of methylated DNA from whole genomic DNA using methylation binding protein (MBP) and hybridization with a DNA array containing complementary oligos with regions of the corresponding promoter gene. The membrane consist of sets of genes, representing specific cellular functions such as tumor suppressor, transcription factor, cell cycle, angiogenesis etc. This is a rapid method designed to detect methylation status of 82 of genes at one time, using less amount of DNA as compared to other methods of methylation analysis such as methylation specific PCR (MSP) and bisulfite sequencing (10,11). Thus, this high throughput screening method has distinct advantage in analyzing abnormal gene expression in various human cancers and diseases. It will help in identifying epigenetic markers involved in cancer development and progression and also provide targets for epigenetic therapy.

The molecular events involved in neoplastic initiation and progression of prostate cancer is poorly understood, despite the recognition of various events during prostate cancer tumorigenesis. Each cancer cell type has several methylated genes but the methylation pattern of individual type of tumor is different (12). In prostate cancer, most of the studies reported methylation in genes such as GSTP1, APC, RASSF1A, RARb2, CRBP1, TIMP3, MGMT, PTGS2 and their association with progression of prostate cancer (13–17). These genes can be seen as only a partial picture of the methylation changes, there may be many more genes which need to be deciphered.

Our data demonstrated methylation of various tumor suppressor genes such as TMS1, RB, RBL1, PAX6, FHIT, DAPK, SRBC and SFN. TMS1, RBL1, DAPK, SFN and RB. These genes were highly methylated in both androgen sensitive as well as in androgen independent cells. TMS1 (Target of Methylation induced Silencing), also known as ASC (Apoptosis Speck like protein containing a CARD) encodes for a CARD (caspase recruitment domain) containing

regulatory protein and has been shown to promote apoptosis directly and by activation of downstream Caspases (18). Das et al (2006) demonstrated that methylation-mediated silencing of TMS1/ASC is a frequent event in prostate cancer (19). RB, RBL1, DAPK and FHIT are tumor suppressor genes showed mild to high methylation in cancer cell lines and in prostate cancer tissues. SRBC is a newly identified tumor suppressor gene which is also hypermethylated in prostate cancer cell lines. Various studies reported the hypermethylation of p27/KIP1, p21 and CDKN2A in prostate cancer (20,21). Our study compliments these observations.

Angiogenesis is an important step in tumor progression. We observed a high to moderate methylation in angiogeneic genes PAI-1, TIMP3, THBS2, and TSP1. Yegnasubramanian et al (2004) also reported hypermethylation in TIMP3 in PC3 and DU145 cell lines (22). Kang et al (15) observed a moderate methylation in THBS1, thus supporting our results.

Our finding suggests a greater hypermethylation in transcription factor genes: STAT1, ATF2, HOXA2, MYOD, STAT5 and SYBL1 in prostate cancer cells. Although there are no reports on STAT1 methylation in prostate cancer but a promoter hypermethylation was observed in squamous cell carcinogenesis (23). POU3F1, NPAT and CIITA were also moderately methylated in prostate cancer cell lines. Epigenetic regulation of MYOD in colorectal cancer (24) and CIITA (25) has been reported but no study reported the methylation in POU3F1, NPAT, STAT5 and HOXA2 so far in prostate cancer.

Membrane transporters and metabolizing genes also play a major role in transport of drugs across the plasma membrane and their subsequent metabolism. We observed more than 10 fold of methylation in MDR1 gene in AR negative cell lines (DU145 and PC3). MDR1 is a well known multi drug resistant gene. Our study confirms other studies that demonstrated hypermethylation of MDR1 in prostate cancer (22,26). MDR1 regulates the trafficking of drugs, peptides, xenobiotics, and ions across cell membranes. Its expression correlates with resistance to hormone therapy and is thought to be important in the progression of primarily hormone-sensitive malignancies like prostate cancer. CFTR, MTX, GPC3 and MLC1 were also found to be methylated in our study. As far as metabozing genes are concerned most of the studies found hypermethylation in GSTP1 gene in 90% prostate tumors (15–17,22). Our MSP and bisulfite sequencing data also confirms methylation of GSTP1 in all three cancer cells but not in normal prostate cells RWPE1. In addition, we observed the methylation in G6PD gene, which is associated with glucose metabolism.

Our data demonstrate methylation of immune related genes and hormone receptors such as IFN-γ, IL4, BAGE, GAGE1, KIR2DL4, LAGE1 and PR in prostate cancer cells. Other interesting genes such as RIOK3, NES-1 and POMC were hypermethylated in prostate cancer cells. However, their exact function needs further elucidation. The biochemical and physiological significance of methylation of these genes in prostate cancer needs to be determined.

We also observed differences in methylation pattern between two AR-independent cell lines (DU145 and PC3). These differences may be associated with their tumor signatures based on their origin, individual phenotype and genotype. Differential methylation pattern between androgen-sensitive and androgen-independent cancer cell suggest an important avenue for targeting receptor associated genes in cancer. Wang et al (2005) also observed a difference in methylation pattern of TGFBR2 between LNCaP and PC3 cell lines (27). Yamada et al (2004) reported a difference in methylation pattern in MFPC7 gene between LNCaP and DU145 (28). A study by Yu et al (2005) in prostate cancer samples and LNCaP, DU145, PC3 cell lines also observed a dramatic difference in methylation pattern (29). Thus, our study compliments these reports demonstrating a difference in methylation pattern between androgen-sensitive vs

androgen-independent cell lines. In contrast, Yegnasubramanian et al (2004) analyzed methylation pattern by Real-Time Methylation- Specific PCR (RT-MSP) in prostate tissues and cell lines. These authors observed no significant difference in methylation pattern between various genes in different prostate cancer cell lines (22). However, we observed some differences between two androgen independent prostate cells PC3 and DU145. Yu et al (29) also observed a difference in methylation pattern between PC3 and Du145 cell lines in few genes such as CSPG4, CTDP1, DSIN, however, they did not analyze their data quantitatively.

In conclusion, our study identified various candidate genes belonging to tumor suppressor, cell cycle, transcription factor and angiogenesis by a promoter methylation array that allows the rapid detection of methylation status of 82 genes at the same time. Identification of differences in methylation pattern between androgen-sensitive and androgen-independent prostate cells may provide opportunities to investigate mechanisms of target genes in the diagnosis, prognosis and treatment of prostate cancer patients. However, additional investigations on the role of DNA methylation and its affect on transcriptional and translational level of regulatory genes should be performed on prostate cancer and normal tissues from patients. An important limitation of our study is analysis of methylation profile in in-vitro ATCC cells. Methylation pattern may vary in patient's samples which are influenced by a variety of additional factors such as age, ethnicity, tumor stage, disease progression and treatment. Hence, there is a need for confirming methylation status of these genes in prostate cancer specimens. Further study on tissue specimens and correlation of the pattern of methylation of these genes with pattern of gene expression silencing may help to define the underlying mechanism of tumor development and switching of prostate cells from AR-sensitive to AR-independent. Thus, the identification of methylation pattern in different genes may open opportunities for epigenetic therapy. Regulation studies confirmed that inhibition of DNA methylation by AZC can restore target gene and its protein expression. As an example, we investigated the expression of MDR1, an important multidrug transporter. MDR1, which was lost in prostate cancer cells, was restored upon AZC treatment, and these cells became more sensitive to growth inhibition by docetaxol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviation list

AR	Androgen Receptor
MBP	Methylation binding protein

Acknowledgments

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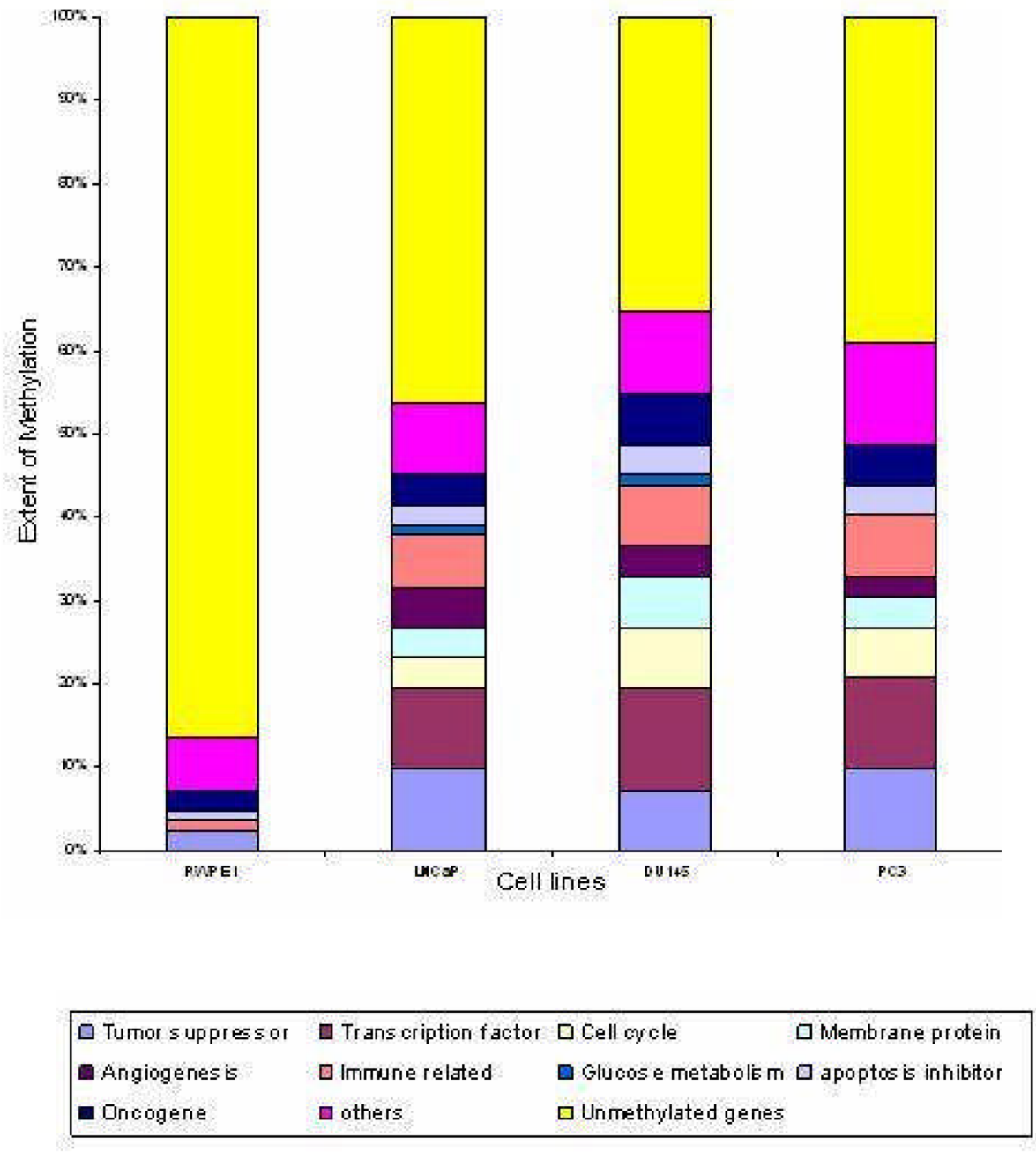

Figure 1.

Frequency distribution of methylated and unmethylated genes in different prostate cancer cell lines. We have taken a volume density (extent of methylation) of 0.5 as cut off to compare the methylation changes. The yellow color bar in each cell line is showing the percentage of unmethylated genes and the rest part is showing the distribution of various sets of methylated genes according to their function.

Figure 2 (A):

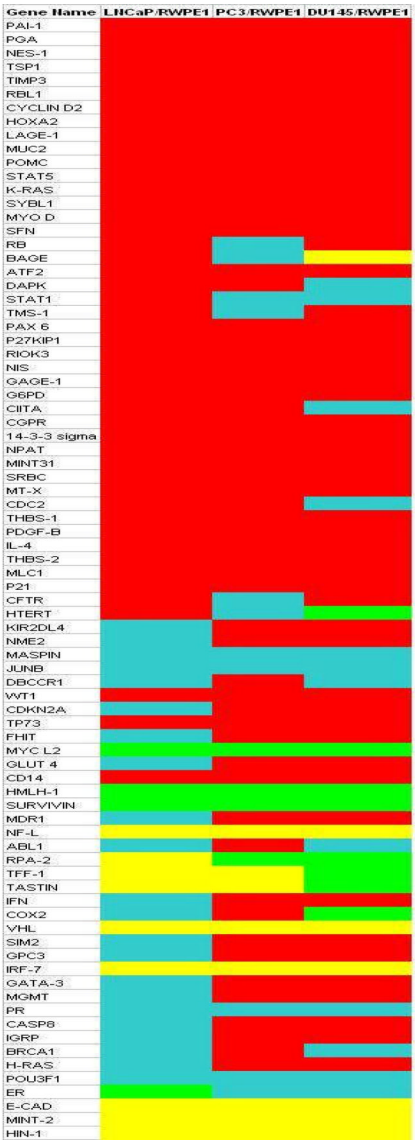

Figure 2 (B):

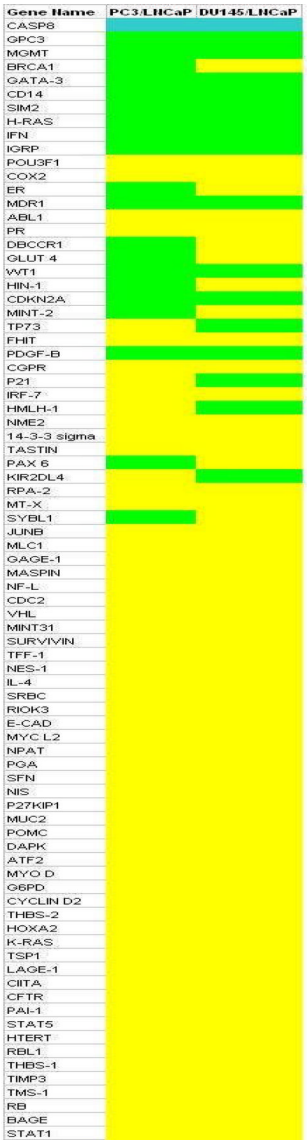

Figure 2.

(A): Difference in methylation pattern of genes between normal vs androgen independent prostate cancer cells. The intensity of methylation for each gene was corrected to its expression level in non cancer prostate cell i.e. RWPE1.

(B) Difference in methylation pattern of genes between androgen dependent (LNCaP) vs androgen independent prostate cancer cells (PC3 and DU145). The alteration in intensity of methylation for each gene was calculated by dividing mean normalized OD of gene in AR negative cell lines (PC3 or DU145) by normalized OD of gene in AR positive LNCaP) cell lines. The extent of methylation is grouped under four categories. These include: <2-fold; 2–5 fold; 5–10 fold; and >10-fold

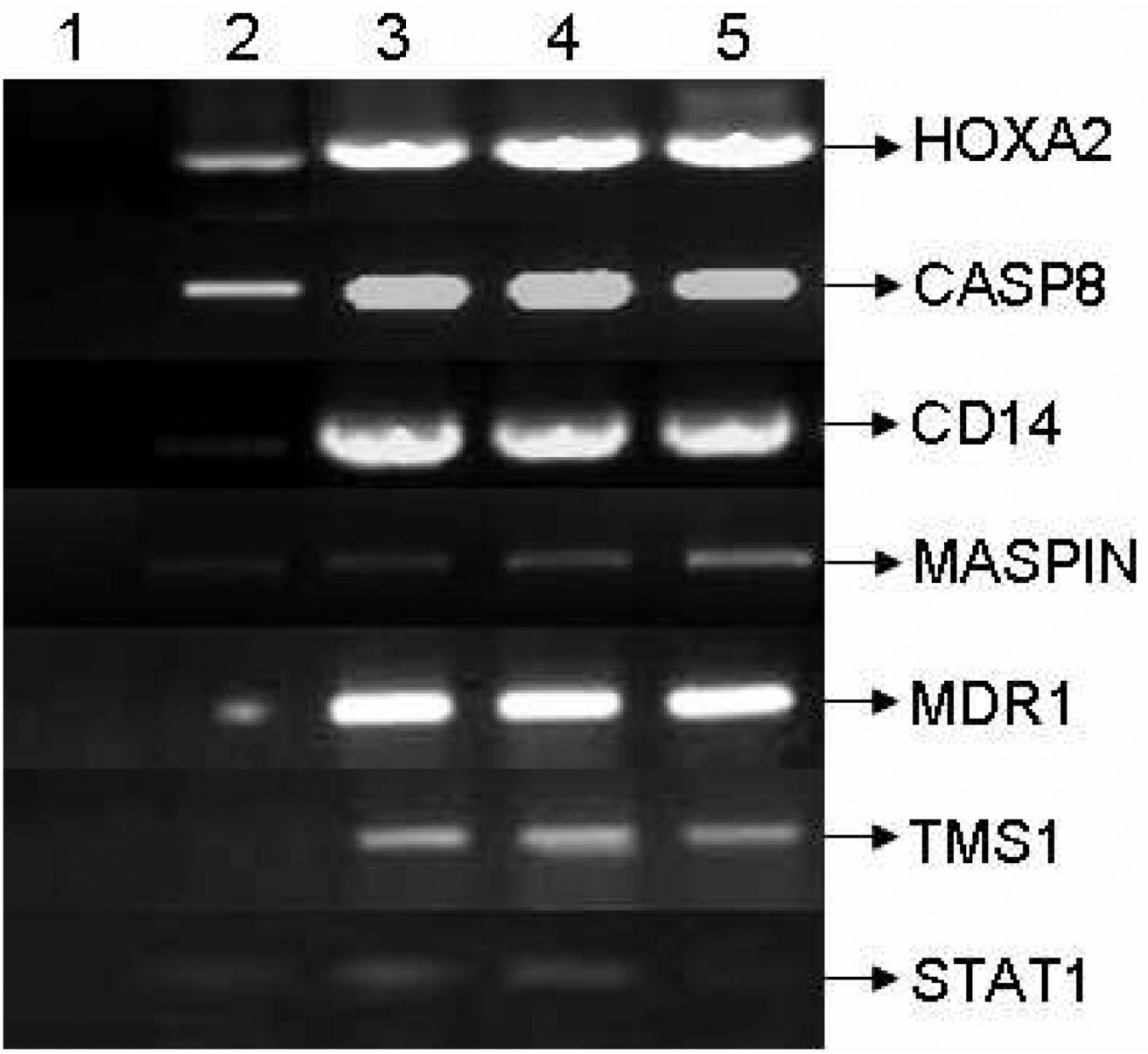

Figure 3.
Promoter Methylation PCR by Panomics methylation PCR kit. According to the kit protocol equal amount of DNA was first digested with Mse1 restriction enzyme and then methylated fragments were purified using methylation binding protein (MBP) and separation column. Equal amount of methylated DNA templates were amplified with gene specific primer in different cell lines.
Lane 1: H_2O; Lane 2: RWPE1; Lane 3: LNCAP; Lane 4: DU145; Lane 5: PC3.

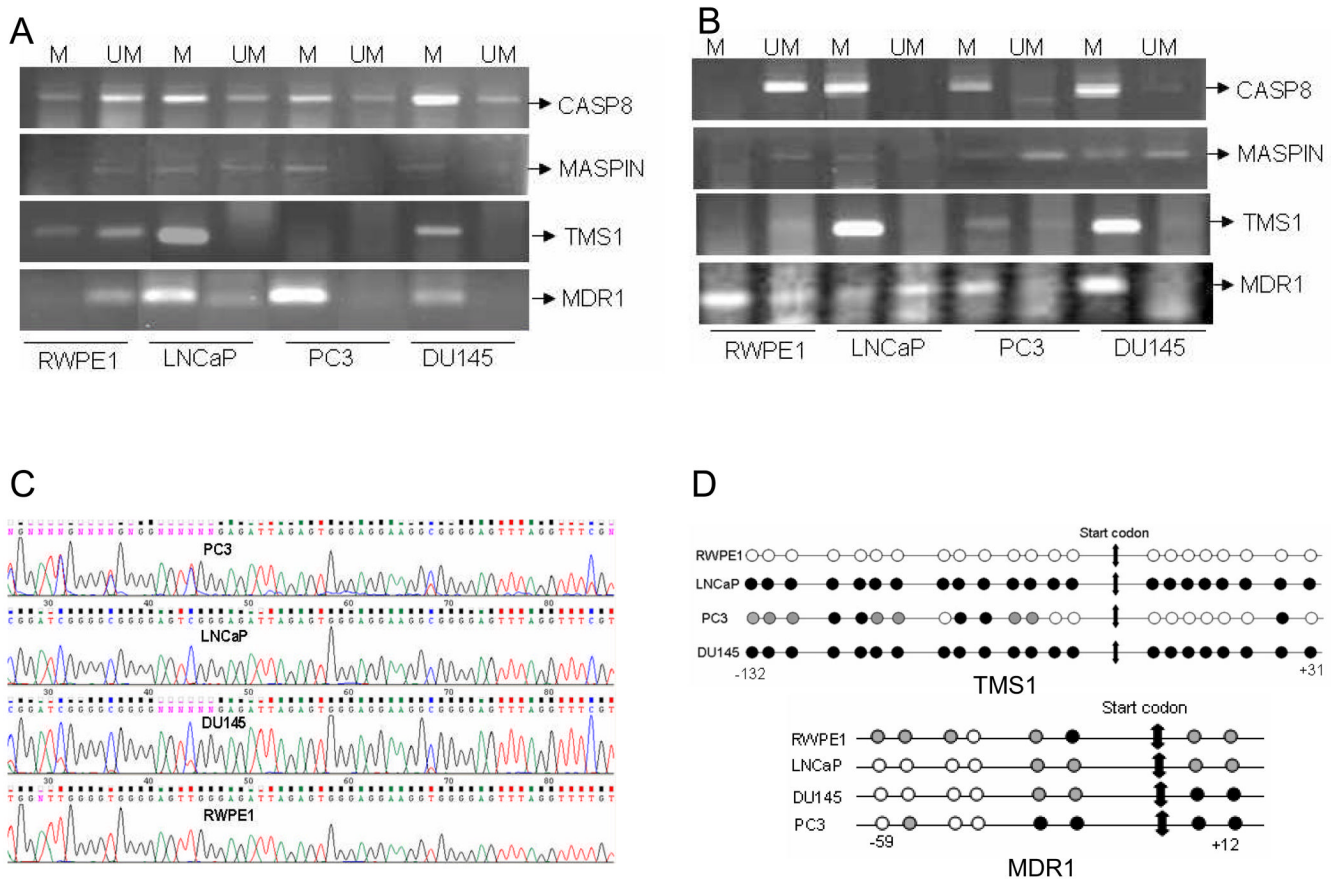

Figure 4.

(A): Methylation specific PCR of CASP8, MASPIN, TMS1 and MDR1 in prostate cancer cells. DNA from prostate cancer cells were Bisulfite modified using EZ methylation kit (Zymo Research) and then PCR done using methylation specific primers and unmethylation specific primers. Because in the cells sometimes methylated and unmethylated both copies are present, so depending upon the number copies they get amplified. **(B)**: Methylation specific PCR of CASP8, MASPIN, TMS1 and MDR1 in 5-aza-dC (5μM/48hrs) treated prostate cancer cells. **(C)** Bisulfite sequencing chromatogram of TMS1 gene showing methylation in LNCaP, DU145 and PC3, partial methylation in PC3 and Unmethylation in RWPE1. **(B)**: Diagrammatic representation of methylation pattern of TMS1 and MDR1 gene at different promoter positions in RWPE1, LNCaP, DU145 and PC3. Dark circles (●) represents methylated sites, shadowed circles (◍) represent partially methylated sites and blank circles (○) represents unmethylated sites in TMS1 and MDR1 promoter region.

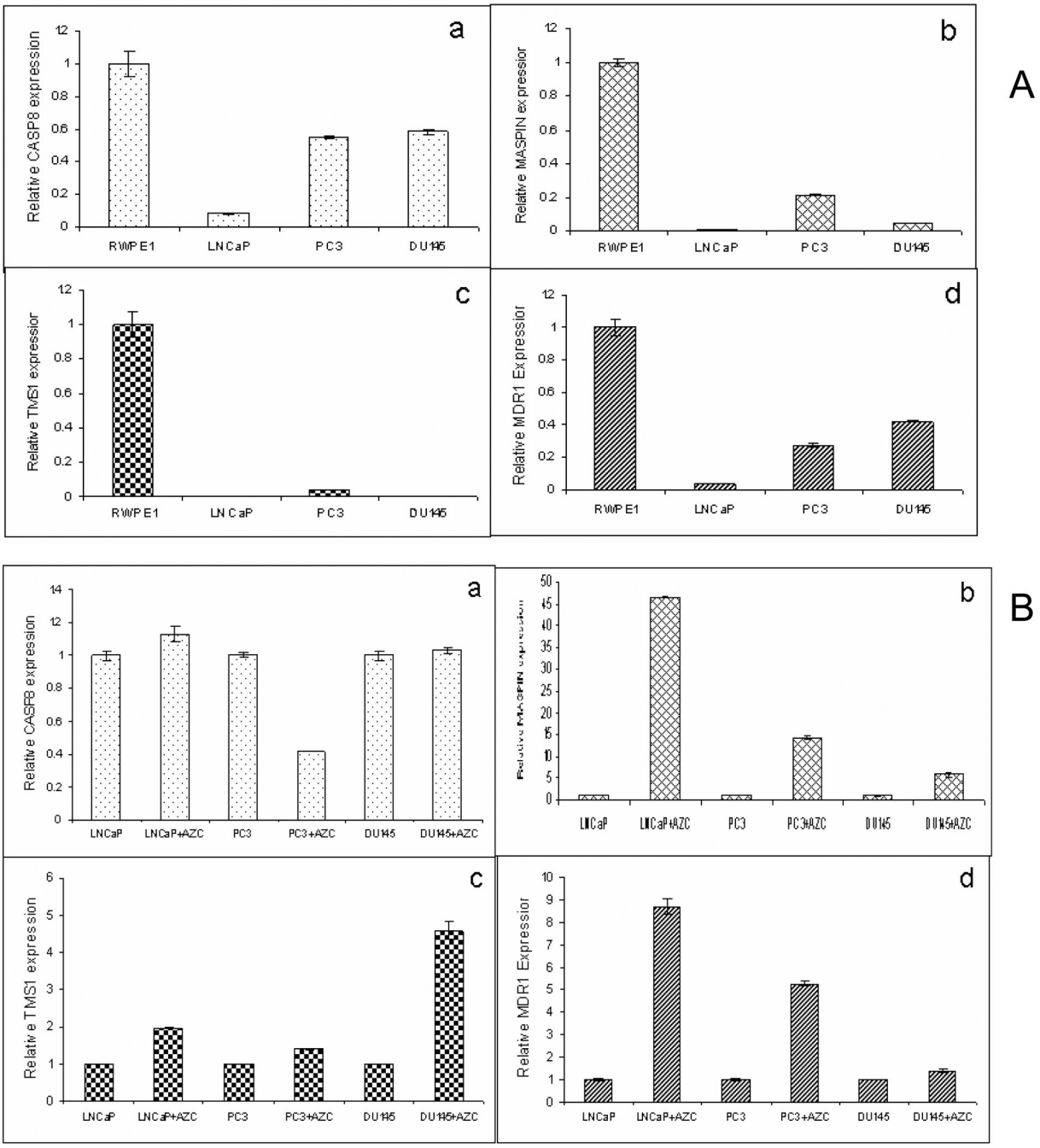

Figure 5.

(A) Relative mRNA expression (mean±SD) of CASP8, TMS1, MDR1 and MASPIN in RWPE1, LNCaP, PC3 and DU145 cell lines. **(B)** Relative mRNA expression (mean±SD) of CASP8 (A), TMS1 (B), MDR1 and MASPIN (D) in untreated and 5-aza-dC (5μM/48hrs) treated prostate cancer cell lines. Each gene is showing an increased expression of respective mRNA except CASP8, which showing a decreased expression.

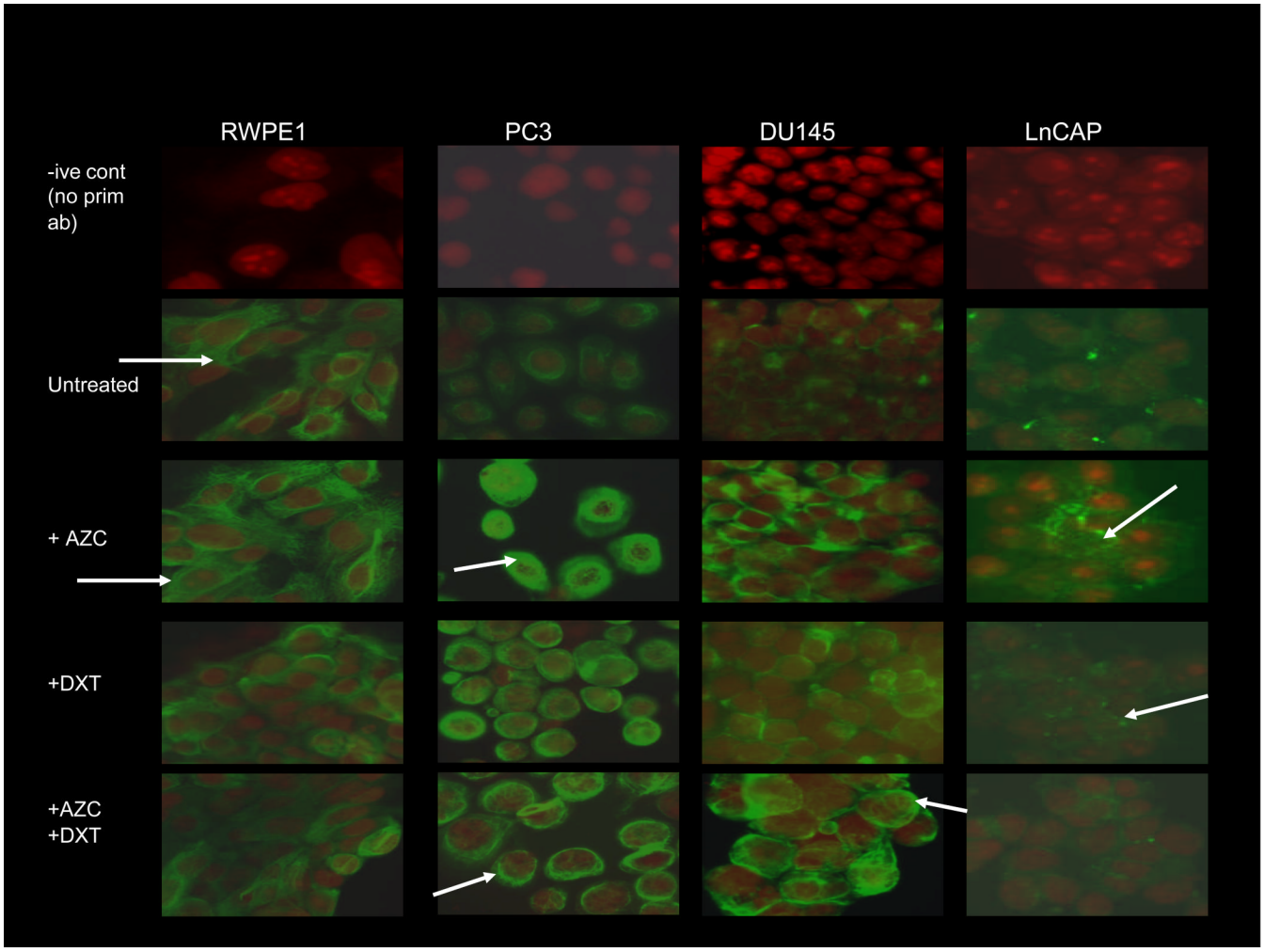

Figure 6.

AZC and docetaxol treatment regulates MDR1 protein expression in normal and cancer prostate cells. Non-tumorigenic prostate cells RWPE1, and prostate cancer cells PC3, DU145, and LNCaP were treated with 5uM of AZC overnight. The next day, monolayer culture were washed and treated further with or without 100 nM docetaxol for 24 hrs. The cell cultures were washed with PBS and fixed with 2% paraformaldehyde containing 0.1% Triton. Immunohistochemistry shows propidium iodide (red) labeled nuclei and green (FITC) labeled MDR1 (Mdr (G-1), monoclonal antibody SC-13131 from Santa Cruz Biotechnology) in the cell membrane or cytoplasm. The data show that untreated RWPE1 express normal levels of MDR1(white arrows), while the prostate cancer cells PC3, DU145, and LNCaP express low levels of MDR1. This is consistent with the loss of MDR1 mRNA in these cells. However, treatment with AZC, restored significant levels of MDR1 in PC3 and DU145, but not in LNCaP. Subsequently, further treatment with docetaxol caused more MDR1 expression in the membranes of PC3 and DU145, but not for LNCaP. The figures are merged data (FITC +propidium red) at x 40.

Table 1

A representative table showing name of the Gene promoters immobilized on the methylation array membrane. Each gene promoter is present in duplicate. (PC- positive control, NC- Negative control)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A	14-3-3σ	14-3-3 σ	ABL1	ABL1	ATF2	ATF2	BAGE	BAGE	BRCA1	BRCA1	Calciton in CGPR	Calciton in CGPR	CASP8	CASP8	CD14	CD14	**PC**
B	CDC2	CDC2	CDKN2A	CDKN2A	CFTR	CFTR	CIITA	CIITA	COX2	COX2	Cyclin D2	Cyclin D2	DAPK	DAPK	DBCCR1	DBCCR1	**PC**
C	E-CAD	E-CAD	ER	ER	FHIT	FHIT	G6PD	G6PD	GAGE1	GAGE1	GATA-3	GATA-3	GLUT4	GLUT4	GPC3	GPC3	**PC**
D	HIN-1	HIN-1	hMLH1	hMLH1	HOXA2	HOXA2	H-Ras	H-Ras	hTERT	hTERT	IFN	IFN	IGRP	IGRP	IL-4	IL-4	**PC**
E	IRF7	IRF7	JUNB	JUNB	KIRDL4	KIRDL4	K-Ras	K-Ras	LAGE-1	LAGE-1	Maspin	Maspin	MDR1	MDR1	MGMT	MGMT	**PC**
F	MINT1	MINT2	MINT31	MINT31	MLC1	MLC1	MT-X(I &II)	MT-X(I &II)	MUC2	MUC2	MYCL2	MYCL2	MyoD	MyoD	NES-1	NES-1	**PC**
G	NF-L	NF-L	NIS	NIS	NME2	NME2	NPAT	NPAT	p21	p21	P27KIP1	P27KIP1	PAI-1	PAI-1	PAX6	PAX6	**PC**
H	PDGF-B	PDGF-B	PgA	PgA	POMC	POMC	POU3F1	POU3F1	PR	PR	Rb	Rb	RBL1 (p107)	RBL1 (p107)	RIOK3	RIOK3	**PC**
I	RPA2	RPA2	SFN	SFN	SIM2	SIM2	SRBC	SRBC	STAT1	STAT1	STAT5a	STAT5a	survivin	survivin	SYBL1	SYBL1	**PC**
J	Tastin	astin	TFF1	TFF1	THBS1	THBS1	THBS2	THBS2	TIMP-3	TIMP-3	TMS-1	TMS-1	TP73	TP73	TSP-1	TSP-1	**PC**
K	VHL	VHL	WT1	WT1	**NC**	**NC**	**NC**	**NC**	**NC**	**NC**	**NC**	**NC**	**NC**	**NC**	**NC**	**NC**	**PC**
L	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**	**PC**

Table 2

Promoter methylation specific primers for different genes used to confirm the methylation status in prostate cancer cell lines.

Gene Name	Primer Sequences (5'-3')
MDR1 Sense	CGTCCTACACCTTAGCAAAAAGA
MDR1 Antisense	GGCAGGCTTGAAAGCACTAA
TMS1 Sense	CAAGCCCAGAGACAAGCAG
TMS1 Antisense	AGCAAAAGGCGCTTCCTTAC
STAT1 Sense	GTTCCCTGGGTTTAGCAACA
STAT1 Antisense	GGGAACTGGCGTTCTGTTTA
CASP8 Sense	TGAGAGAACAGGGGAGGGTCTAG
CASP8 Antisense	CATGGACGTGCAAACTAAAGCC
CD14 Sense	GAGGATATTCAGGGACTTGGATTTG
CD14 Antisense	GGTCGATAAGTCTTCCGAACCTCT
MASPIN Sense	CAAGAGGCTTGAGTAGGAGAGG
MASPIN Antisense	TGGAGTCACAGTTATCCTGGAA
HOXA2 Sense	TGGGCCCGGGGCGCAGACTCTGG
HOXA2 Antisense	GCAGGAGAAAGGAGCAGAGGAA

Table 3

Details of some candidate hypermethylated genes in AR positive and AR negative prostate cancer cell lines as compared to normal cell line.

Gene Name	Methylation Fold in AR positive (LNCaP) compared to RWPE1	Mean±SD methylation Fold in AR negative compared to RWPE1	Description of Gene	Chromosome location
Tumor Suppressor Genes				
TMS-1	17.7	9.47±3.68	Target of methylation – induced silencing 1	16p12-p11.2
14-3-3 sigma	16.7	15.42±2.80		1p36.11
RB	19.2	19.62±3.55	Retinoblastoma	13q14.2
RBL1	20.9	14.64±1.61	Retinoblastoma like 1	20q11.2
PAX6	12.2	17.81±2.12	Paired box gene 6	11p13
DAPK	18.7	10.89±5.86	Death associated protein kinases	9q34.1
SRBC	12.4	12.94±1.12	Protein kinase C, delta binding protein	11p15.4
MASPIN	6.2	6.68±0.91	protease inhibitor 5	18q21.3
Transcription Factors				
STAT1	17.6	7.59±2.47	Signal transducer and activator of transcription 1	2q32.2
ATF2	19.7	14.37±0.27	Activating transcription factor	2q32
MYOD	15.9	16.62±1.62	myogenic differentiation 1	11p15.4
SYBL1	13.2	18.56±5.24	Synaptobrevin-like 1	Xq28 Y28
NPAT	15.9	14.19±1.64	Nuclear protein, ataxia-telangiectasia locus	11q22-q23
STAT5	22.5	15.68±5.05	Signal transducer and activator of transcription 5A	17q11.2
CIITA	16.3	9.36±0.87	class 2 MHC transactivator	16p13
HOXA2	21.8	21.75±7.21	Homeobox A2	7p14p15
GATA3	6.4	15.11±0.57	Gata binding protein	10p15
SIM2	7.6	15.87±1.55	Single minded homologue 1	21q22.13
WT1	10.2	17.46±2.08	Wilms tumor1	11p13
Cell Cycle genes				
p27KIP1	17.0	17.35±4.65	Cyclin-dependent kinase inhibitor	12p13.1-p12
CDC2	12.9	10.65±2.69	Cell division cycle 2	10q21.1
CyclinD2	22. 4	16.33±1.19	Cyclin D2	12p13
CDKN2A	6.2	17.56±4.25	cdk inhibitor 2a	9p21
p21	11.8	17.70±2.90	cyclin-dependent kinase inhibitor 1A	6p21.2
K-RAS	21.6	16.89±2.84	Kirsten rat sarcoma virus oncogene	12p12.1
TP73	11.1	14.99±3.84	Tumor protein p73	1p36.3

Gene Name	Methylation Fold in AR positive (LNCaP) compared to RWPE1	Mean±SD methylation Fold in AR negative compared to RWPE1	Description of Gene	Chromosome location
Apoptotic genes				
CASP8	6.4	34.18±4.37	Caspase 8	2q33-q34
CD14	11.4	35.51±3.79	CD14	5q31.1
Membrane proteins				
MDR1	6.2	13.66±0.56	Multiple drug resistant 1 (p-glycoprotein)	7q21.1
MLC1	11.4	14.07±2.29	Megalencephalic leukoencephalopathy with subcortical cysts 1	22q13.33
CFTR	10.9	8.45±1.90	cystic fibrosis transmembrane conductance regulator	7q13.2
MTX	14.4	16.49±3.97	Metaxin	1q21
GPC3	5.7	17.89±3.72	Glypican 3	Xq26.1
GLUT4	8.6	12.25±1.09	Solute carrier insulin transport	17p13
Glucose Metabolism				
G6PD	14.2	12.19±1.89	Glucose-6-phosphate dehydrogenase	Xq28
DNA repair gene				
MGMT	7.0	17.73±2.90	O-6-methylguanine-DNA methyltransferase	10q26
Immune related				
IFN	6.7	16.37±1.52	Interferon	9p22
LAGE-1	22.98	14.53±2.00	CTAG2/cancer/testis antigen 2	Xq28
GAGE 1	17.2	17.51±3.16	G antigen 1	Xp11.4-11.2
IL4	11.2	11.60±0.43	Interleukin 4	5q31.1
PDGF-B	10.7	19.93±1.43	Platelet-derived growth factor beta polypeptide	22q13.1
KIRDL4	8.7	12.08±3.31	Killer cell immunoglobulin like receptor 2DL4	19q13.4
Angiogenesis Inhibitors				
TIMP3	22.3	12.47±3.44	Tissue inhibitor of metallopeptidases-3	22q12.3
PAI-1	26.8	21.76±3.53	Plasminogen activator inhibitor 1	7q21.3-q22
TSP1	23.01	15.58±3.27	Thrombospondin 1	15q15
Other genes				
RIOK3	11.6	13.06±3.12	RIO kinase 3, Serine threonine kinase activity, nucleotide binding	18q11.2
NIS	13.3	13.32±0.34	sodium-iodide symporter	19p13.2-p12
POMC	20.5	19.89±1.57	Proopiomelanocortin	2p23.2