Sex steroid-induced DNA methylation changes and inflammation response in prostate cancer.

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Abstract

Background: sex steroid hormones have been reported to induce inflammation causing dysregulation of cytokines in prostate cancer cells. However, the underlying epigenetic mechanism has not well been studied. The objective of this study was to evaluate the effect of sex steroid hormones on epigenetic DNA methylation changes in prostate cancer cells using a signature PCR methylation array panel that correspond to 96 genes with biological function in the human inflammatory and autoimmune signals in prostate cancer. Of the 96-gene panel, 32 genes showed at least 10% differentially methylation level in response to hormonal treatment when compared to untreated cells. Genes that were hypomethylated included *CXCL12*, *CXCL5*, *CCL25*, *IL1F8*, *IL13RA1*, *STAT5A*, *CXCR4* and *TLR5*; and genes that were hypermethylated included *ELA2*, *TOLLIP*, *LAG3*, *CD276* and *MALT1*. Quantitative RT-PCR analysis of select genes represented in a cytokine expression array panel showed inverse association between DNA methylation and gene expression for *TOLLIP*, *CXCL5*, *CCL18* and *IL5* genes and treatment of prostate cancer cells with 5'-aza-2'-deoxycytidine with or without trichostatin A induced up-regulation of *TOLLIP* expression. Further analysis of relative gene expression of matched prostate cancer tissues when compared to benign tissues from individual patients with prostate cancer showed increased and significant expression for *CCL18* (2.6 fold; $p < 0.001$), a modest yet significant increase in *IL5* expression (1.17 fold; $p = 0.015$), and a modest increase in *CXCL5* expression (1.4 fold; $p = 0.25$). In conclusion, our studies demonstrate that sex steroid hormones can induce aberrant gene expression via differential methylation changes in prostate carcinogenesis.

Introduction:

Prostate cancer (PCa) remains a major public health concern for the male population throughout the Western world and is a leading cause of cancer death among men in the United States¹. The role of sex steroid hormones in prostate cancer is well documented (for recent reviews see ^{2,3}). Genes that are involved in the sex steroid hormones and components of their signaling and metabolic pathways have been extensively investigated in relation to prostate cancer susceptibility. In particular, androgen biosynthesis and androgen receptor (AR) mediated signaling are necessary for the growth and development of the prostate from fetal life through adulthood. Allelic frequencies of several genes in androgen signaling pathways have been shown to be associated with prostate cancer development and resistance to therapy⁴. Thus androgen signals are targets in PCa treatment, and the importance of androgen deprivation therapy is well established^{5,6}. There is evidence that elevation of sex steroid hormones (e.g. estrogens) can induce inflammation in the prostate and also dietary carcinogens and inflammatory oxidants have been implicated in inducing damage to the prostate epithelium via inflammation⁷. Thus, it is possible that early inflammatory events stimulated by sex hormones can serve as a prerequisite for the onset of prostate cancer. Studies carried out by De Marzo et al.⁸ described a lesion in the prostate which they termed proliferative inflammatory atrophy (PIA). The PIA has the distinct morphological appearance of prostate atrophy with associated prostatic inflammation suggesting that chronic or recurrent inflammation may be a risk factor in the pathogenesis of prostate cancer⁸. The PIA lesions show many signs of ongoing stress, including induction of oxidative metabolizing enzymes such as the glutathione S-transferases (GSTs) GSTA1 and GSTP1, and of cyclooxygenase-2 (COX-2)⁹. However, in a subset of the PIA lesions, GSTP1 expression is silent, and the mechanism inducing gene silencing is by DNA hypermethylated¹⁰. Furthermore, the silencing of GSTP1 expression persist in prostatic intraepithelial neoplasia (PIN) lesions, later PCa precursors, and in prostatic carcinomas, hinting at some sort of selective growth or survival advantage^{10,11}. Interestingly, DNA methylation of *GSTP1* is absent in normal prostatic epithelial cells, but increases in the normal aging prostate, or in PIA lesions which commonly accumulate in the prostate with age^{10,12}. Since the recognition that the *GSTP1* is frequently hypermethylated in PCa, more than 40 genes have been reported to be targets of DNA hypermethylation-associated epigenetic gene silencing in PCa cells¹³. For human PCa, there is abundant evidence to suggest that somatic DNA methylation may appear earlier during cancer development than genetic changes, as well as more commonly and consistently¹⁴. There is also increasing evidence to suggest that sex steroid hormones can trigger inflammatory induced epigenetic gene silencing in addition to genomic alterations in prostate cancer¹⁵. In PCa, proinflammatory cytokines are reportedly susceptible to altered expression via aberrant DNA hypermethylation promoting carcinogenesis^{16,17}. However, very few studies has been directed to investigating the role of sex steroid hormones induced inflammation and

associated epigenetic changes in prostate cancer. In the present study, we employed the EpiTect Methyl II PCR technology that provides ready-to-use, predesigned primer assays to simultaneously assess the methylation status of multiple inflammatory genes that can be altered by sex steroid hormones in prostate cancer. We report differences in gene promoter and concomitant expression levels of several human inflammatory and autoimmune cytokines signals in association with hormonal induction in prostate cancer cells.

Materials and Methods

Prostate Cell lines and human prostate tissues. The human PCa cell lines; PC3, DU145 and LNCaP were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The immortalized normal prostate epithelial cell line pNT1A was obtained from the European Collection of Cell Culture (Salisbury, U.K.). All cell lines are maintained in complete medium containing RPMI supplemented with fetal bovine serum (10%) (FBS; Invitrogen, Carlsbad, CA) and 1% Penicillin Streptomycin (GIBCO) unless otherwise stated. Human prostate tissues samples were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence (SPORE) in the prostate cancer tissue bank. Fresh frozen tissue punches of normal and tumor tissues were obtained from Baylor SPORE at the time of radical prostatectomy. The pathological status was confirmed before processing, and the tumor samples had a tumor cell percentage of 70%-100% with Gleason scores of 6-8.

Hormonal treatment and cell growth Assay: Cells were seeded on a 6-well plate at 1×10^6 cells per well in complete growth medium. Twenty-four hour later, cultures were replaced with medium containing 2% charcoal-treated FBS to remove steroids, followed by an additional 24 hours in RPMI containing 2% charcoal-treated FBS. The synthetic ligands; R1881 (10^{-11} M, 10^{-10} M), testosterone (10^{-9} M, 10^{-8} M), 17β -estradiol (10^{-9} M, 10^{-8} M), progesterone (10^{-9} M, 10^{-8} M) and dexamethasone (10^{-8} M, 10^{-7} M); were obtained from Sigma Aldrich (St. Louis, MO). Control vehicle (DMSO) treated cells were grown using the same medium without hormones. After 1-3 days in the culture, cell proliferation was assessed by Coulter counter. Experiments were done at least 3 times.

DNA isolation and DNA methylation PCR array: Genomic DNA was extracted from cells using the QIAMP DNA Mini Kit (Qiagen Inc.; Valencia, CA) according to the manufacturer's instructions. The methylation status of selected genes was evaluated using the Methyl-Profiler DNA Methylation PCR Array: MeAH-3520E (Human Inflammatory Response & Autoimmunity DNA Methylation PCR Array signature Panel: SABiosciences, Qiagen; Frederick, MD). As noted by the manufacturer, the Panel profiles the methylation status of 96 gene promoters involved in expression silencing during development

and oncogenesis. Genomic DNA of which 1µg was used in the PCR array analysis conducted according to the manufacturer's protocol. Briefly, the Methyl-Profiler DNA Methylation PCR System is based on restriction enzyme digest and quantitative PCR. DNA methylation-sensitive and methylation-dependent restriction enzymes are used to selectively digest unmethylated or methylated DNA, respectively. The remaining DNA after digestion is quantified by real-time PCR using primers that flank the region of interest. The relative concentrations of differentially methylated DNA (specifically hypermethylated, intermediately methylated, and unmethylated DNA) are determined by comparing the amount in each digest with that of a mock digest (contains all the input genomic DNA without restriction enzymes). The arrays were run on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Grand Island, NY) using one cycle of 10min at 95°C to activate the DNA polymerase, followed by 40 cycles of 97°C for 15s and 72°C for 1min in accordance with specifications in the Methyl-Profiler protocol. Dissociation curve analysis were analyzed to confirm homogeneity of the amplification products.

DNA methylation PCR array Data analysis: The relative amount of methylated DNA fraction is calculated using the difference in threshold cycle (C_T) method, normalizing the amount of DNA in each digest against the total amount of input DNA in the mock digest. The Methyl-Profiler PCR Array Excel-based data analysis was performed using the web-based analysis tool (www.sabiosciences.com/pcrarraydataanalysis.php), which includes descriptive statistics, including fold change, volcano plots, and cluster analyses. The C_T values were entered into the raw data table and results were automatically determined by the template through a series of mathematical equations for each gene analyzed. These equations and calculations are described in detail in the Methyl-Profiler DNA Methylation PCR Array System User Manual (SABioscience, Qiagen). Percent methylation was calculated by these equations using the appropriate C_T values. Heatmap analysis was performed using R statistical software (<http://r-project.org>).

RT-PCR: For the human inflammatory cytokines & receptor RT² ProfilerTM PCR Array (24 gene panel array; SABiosciences, Qiagen; Frederick, MD) analysis; 1.5µg of total RNA was extracted from cultured cells using TRIzol Reagent (Invitrogen) and converted to cDNA using cDNA synthesis kit (SA Biosciences, Frederick, MD). The cDNA was added to RT² qPCR master mix (SA Biosciences, Frederick, MD) and aliquoted across the 24 gene panel PCR array and run using the real-time PCR cycling program according to the manufacturer's instruction (SA Biosciences, Frederick, MD). For individual gene expression analysis by quantitative RT-PCR (qRT-PCR); RNA was isolated from cultured cells or prostate tissues using TRIzol Reagent (Invitrogen) and used in cDNA synthesis using Invitrogen SuperScriptTM (Thermo Fisher Scientific, Waltham MA). The RT-PCR analysis was carried out using Taqman probes designed for gene specific assays (see Table for Taqman assays). Real-time PCR was

carried out in a Bio-Rad iCycler real-time thermal cycler (Hercules, CA) as described previously¹⁸ and incorporating optimized PCR reaction conditions for each gene. The C_t values in log linear range representing the detection threshold values was expressed in ΔC_t (C_t value of target gene minus C_t value of housekeeping β -actin gene).

Treatment with 5-aza-2'-deoxycytidine (5-aza-dC) and/or Trichostatin A (TSA). The LNCaP cells were seeded at 5×10^5 cells/100-mm tissue culture dish. Twenty-four hour later, cultures were replaced with medium containing 2% charcoal-treated FBS, followed by an additional 24 hours in RPMI containing 2% charcoal-treated FBS and R1881 (10^{-10} M) plus 5'-aza-dC (5 μ M) for 96 hours with or without TSA (250 nM) for additional 24 hours. Total RNA extracted from cells using TRIzol Reagent (Invitrogen) was first used in qRT-PCR as described above. Mock-treated cells were cultured similarly without drug treatment. Three independent experiments were carried out for each analysis.

Results:

In order to assess the growth of prostate cancer cells in response to different sex steroid hormones, the androgen-dependent LNCaP prostate cancer cells were treated with 5 different sex steroid hormones in a dose dependent and time dependent manner. The LNCaP cells contain mutated amino acid in the steroid binding domain of the androgen receptor which allows broad steroid binding specificity for progestogens, estradiol and several antiandrogens. Treatment of LNCaP cells with synthetic nonmetabolizable androgen 17 beta-hydroxy-17 alpha-methyl-(3H)-estra-4,9,11-trien-3-one (R1881; 0.1 nM) stimulated cell growth by approximately 2 fold on day 3 of treatment (Figure 1). Whereas a lower concentration of R1881 (0.01 nM) modestly stimulated cell growth. Estradiol (10 nM), testosterone (10 nM), Progesterone (1 nM) did not inhibit androgen responsive growth of LNCaP cells but had similar growth stimulatory effects as R1881 (causing 1.5 fold, 1.5 fold and 1.3 fold induction in growth compared to control respectively). Interesting the combined R1881 and estradiol (0.1 nM each) treatment induced 2 fold in growth stimulation, whereas (1 nM each) induced lesser growth stimulation (1.5 fold) on day 2. However, the longer incubation of LNCaP with both R1881 and estradiol for a 3 day period showed reduced growth stimulation compared to 2 day period of the same induction suggesting that higher concentrations of the combined hormones maybe toxic to LNCaP cell growth. We did not see significant effect of dexamethasone (1 nM or 10 nM) treatment on LNCaP cells proliferation. Overall, the results demonstrate that LNCaP cell proliferation is stimulated by physiological R1881, estradiol and testosterone

concentration. Testosterone is the major exogenous stimulus for the growth of prostatic carcinoma and testosterone can be converted to estradiol-17 β by the enzyme aromatase which is expressed in the human prostate. Thus, estrogen may be involved in prostate cancer induction by testosterone. In addition, estrogen receptors are expressed in the prostate and may mediate some or all of the effects of estrogen. Because LNCaP cells does not possess glucocorticoid receptor¹⁹, the synthetic glucocorticoid; dexamethasone maybe signaling through the mutated androgen receptor in LNCaP cells.

To examine the simultaneous induction of DNA methylation changes in a panel of gene promoters in response to sex steroid hormones, LNCaP cells were exposed to different hormones and at concentrations that produced highest cell proliferation (Figure 1 above; 0.1 nM R1881; 10nM Testosterone; 10nM Estradiol; 1mM and 0.1 nM each of R1881 + Estradiol) or mock control treatment for 72 hours and the degree of DNA methylation evaluated using Human Inflammatory Response & Autoimmunity DNA Methylation PCR Array. The signature panel was chosen based on representation of genes that have been reported to play roles in inflammation and/or immune response. The Methyl-Profiler returns result as the proportion/percentage of densely, intermediately and sparsely methylated target sequences in the DNA samples. A consistent feature of the methylation results observed was that no intermediately methylated sequence was detected in any of the analyzed genes in any hormone treated or the mock control cell cultures. Therefore, the methylation results are presented as proportions of densely methylated sequences, and the rest of the target DNA (1 minus the proportion of densely methylated sequences) is determined as a sparsely methylated or unmethylated molecular population. Of the 96 genes represented on the array panel, 64 demonstrated less than 10% difference in methylation frequency between mock and all hormone treated cells (data not shown). To be more stringent, we focused on the 32 genes which demonstrated 10% or higher differential methylation level in response to treatment of at least one hormone when compared to mock control (Figure 2). Hierarchical clustering analysis showed that the R1881, testosterone, estradiol or the combined R1881 and estradiol treatment clustered together and showed similar methylation pattern whereas treatment with dexamethasone showed different methylation pattern indicating differences in the regulation of genes in response to these hormones.

To ascertain if the methylation changes induced by R1881 treatment is associated with gene silencing in LNCaP cells, we performed gene expression analysis by quantitative RT-PCR using inflammatory cytokine and receptors PCR array (Figure 3). For the methylated genes that were present in the gene expression array we observed an inverse correlation of DNA methylation and gene expression. The TOLLIP gene that was hypermethylated in LNCaP cells treated with R1881 also showed a decrease in expression at the RNA transcript level, whereas CXCL5 gene that were hypomethylated upon R1881 treatment, had increased gene expression compared to control LNCaP cells suggesting that alteration in

methylation at the promoter level may be contributing to changes in gene expression. To further verify the expression levels of these cytokines in the prostate cell lines and tissues, we performed quantitative RT-PCR using Taqman assays designed for 5 of the cytokines (Figure 4). In all the prostate cancer cell lines; LNCaP, PC3 and DU145 examined, we observed an increased expression in CXCL5 when compared to the normal primary epithelial cells (pNT1A; Figure 4a). We also observed higher expression for IL5 and CCR1 in the androgen-dependent LNCaP cells when compared with pNT1A cells. The androgen-independent cell lines; PC3 and DU145 showed higher gene expression for CXCL5 gene and lower expression for IL5 genes in comparison with pNT1A or LNCaP cells. In addition, DU145 showed higher expression for CCR1 when compared with the other cell lines. Overall our observations suggest that R1881 can induce changes in cytokine expression via DNA methylation in prostate cancer cells even in the absence of androgen receptor (PC3 and DU145).

To further assess if the expression level of these cytokines in prostate cell lines correlate with prostate tissues expression, we measured the mRNA transcript level of 5 genes represent in the expression array: CXCL5, LTB4R, CCR1, CCL18 and IL5 in matched normal and neoplastic prostatic tissues using quantitative RT-PCR analysis with mRNA from a total of 40 pairs of matched normal and tumor prostate tissue samples. We used β -actin as an endogenous mRNA control. The real-time data is presented as the ratio of each gene X $10^3/\beta$ -actin transcript for the samples analyzed (figure 4b). The expression of each gene in both normal and cancer tissues were variable, presumably reflecting both random variability in tissue composition (i.e., epithelial content) and variable expression per cell. We observed a modest and non-significant increase in gene expression in the cancer tissues when compared to normal prostate tissues for CXCL5 (1.4 fold; $p = 0.25$) and CCR1 (1.15 fold; $p = 0.94$) genes. On the other hand, we observed a significantly higher gene expression for CCL18 (2.6 fold; $p < 0.0001$) and IL5 (1.17 fold higher; $p = 0.015$) genes. In addition, we observed a significant but a modest reduction in LTB4R (0.903 fold; $p = 0.037$) gene expression in cancer samples compared to normal tissues.

To verify if the methylation status of these genes affects expression, we tested the hypothesis that pharmacological modulation of methylation can reactivate gene expression²⁰. The LNCaP cell line was treated with the demethylating agent 5'-Aza-2'-deoxycytidine (5'-Aza-dC) with or without the histone deacetylase inhibitor, trichostatin A (TSA), and gene expression analyzed by quantitative RT-PCR using untreated and treated cells to assess whether promoter CpG methylation was closely associated with gene expression in a set of genes (Figure 4c). Treatment with 5'-Aza-dC caused more than 2 fold increase in expression for TOLLIP gene and a modest induction for IL5, CXCL5, CCL18 LTBR4 genes, whereas there was a reduction in gene expression for CCR1. The combined of 5'Aza-dC and TSA treatment synergistically increased mRNA transcript for TOLLIP gene only. The combined 5'Aza-dC and TSA caused modest increased in IL5 and CCL18 genes, whereas it caused inhibition of CCR1, CXCL5 and

LTBR4 expression. The result shows that treatment with 5'Aza-dC and TSA is able to induce the re-expression of hypermethylated TOLLIP gene in prostate cancer cells. The expression levels of CCL18²¹ and LTBR4²² has been reported to be increased in prostate cancer and maybe associated with disease progression, whereas one report of differential expression of IL5²³ and risk of lung cancer in different racial and ethnic group. Overall, our studies demonstrates an inverse correlation of cytokine expression and DNA methylation and that pharmacological drugs are able to modulate gene expression suggesting a potential role for epigenetic DNA methylation and histone modification in the regulation of several cytokines including *CCR1*, *IL5*, *CXCL5*, *CCL18*, *TOLLIP*, and *LTBR4* genes in prostate cancer cells.

Discussion:

Inflammation is a complex immune response that is mainly mediated and regulated by inflammatory cytokines. Several proinflammatory genes including members of the interleukin family play roles in apoptosis, angiogenesis, and proliferation. One potential mechanism whereby sex steroid hormones can modulate the expression of cytokines and their receptors is by activating the expression of transcription factors such as nuclear factor kappa B (NF- κ B). It is hypothesized that elevated estrogen, in the presence of testosterone, causes prolonged activation of a redox-sensitive transcription factor, nuclear factor kappa B (NF kappa B), that initiates and amplifies an inflammatory cascade within the prostate and results in sustained oxidative and nitrative damage²⁴. Furthermore, overexpression of NF- κ B becomes constitutively active in most tumors²⁵. Additional recent studies show that chemokines and their receptors play a critical role in prostate cancer development and progression in addition to their role in development and inflammatory responses²⁶. However, few studies have investigated a role of epigenetic mechanism in regulating cytokine expression in prostate cancer. Joyce et al.²⁷ have reported that hypermethylation of inflammatory genes, specifically *IFN* and *ICAM-1* were associated with prostate carcinogenesis. Another report has shown that high expression of CD133 by promoter demethylation may contribute to the resistance of tumor cells to chemotherapy and apoptosis²⁸.

In the present study, we have studied DNA methylation as a potential mechanism in regulating the expression of inflammatory cytokines in response to sex steroid hormones stimulation of androgen dependent prostate cancer cell line, LNCaP. The LNCaP cells contain an abnormal androgen receptor system with broad steroid binding specificity for progestogens, estradiol and several antiandrogens²⁹. The mutated androgen receptor of LNCaP cells is therefore a useful tool in the elucidation of different levels of action of steroids and anti-steroids. We analyzed DNA methylation changes using genomic DNA extracted from LNCaP cells that has been treated with different sex steroid hormones in a PCR reaction of promoter gene array. For this study, we used the Human Inflammatory Response & Autoimmunity DNA

Methylation PCR Array signature Panel Arrays that allows the simultaneous detection of the methylation status of 96 genes that are either tumor suppressor genes or key genes involved in the inflammatory response and autoimmunity pathways. Using this approach, we have detected differential methylation of several cytokines and their receptors, and other proinflammatory genes in response to sex steroid hormone stimulation of the prostate cancer cell line.

Cytokines and their receptors have been reported to play roles tumorigenesis; the interaction between CXCL12 and CXCR4 promotes cellular adhesion, survival, proliferation, and migration. We have observed hypomethylation of several chemokines including *CXCL5*, *CXCL3*, *CXCL1*, and the *CXCL12* receptor *CXCR4* in response to R1881, Estradiol, and Testosterone induction of LNCaP cells. Interestingly, we observed hypermethylation of *CXCL12* promoter only in the R1881 treated cells whereas all the other hormones caused hypomethylation of the *CXCL12* promoter. One report has suggested hypermethylation of *CXCL12* to be associated with advanced breast cancer³⁰. Differential regulation CXCR4 expression by DNA methylation has been reported in different tissues. The CXCR4 gene is upregulated in skin, lung, pancreas, brain and breast cancer tissues, whereas in pancreatic cancer and melanoma, CXCR4 is down-regulated by hypermethylation³¹. Another report observed both *CXCL12* hypermethylation and *CXCR4* unmethylation in breast cancer cells and this was correlated with shorter overall survival and disease-free survival³². The serine protease elastase 2 (*ELA2*) may play a role in degenerative and inflammatory diseases by its proteolysis of collagen-IV and elastin of the extracellular matrix. We observed moderate methylation of *ELA2* gene in response to R1881 or testosterone treatment, whereas estradiol or the combined treatment of R1881 and estradiol treatment showed low methylation. There is at least one report that also suggest moderate to high DNA methylation *ELA2* in CML cell lines³³. The Toll-interacting protein (*TOLLIP*) is one well known regulator of TLR signaling. High expression of *TOLLIP* can provide a negative feedback loop for TLR-mediated inflammation to protect cells. We observed that in prostate cancer cells *TOLLIP* is hypermethylation in response to sex steroid hormone treatment whereas the pharmacological modulation with demethylating agent and histone deacetylase inhibitor is able induce *TOLLIP* gene expression demonstrating that epigenetic mechanism is playing a role in the regulation of *TOLLIP* in response to androgen signal. In support of our observation, one group has reported that silencing of *TOLLIP* rescued TLR-triggered cytokine production by the histone lysine methyltransferase *Ezh1*³⁴ and this may contribute to prostate cancer progression. Several reports have demonstrated lower expression of *TOLLIP* in cancer tissues in comparison with benign tissues suggesting that DNA hypermethylation may play a potential role in regulating *TOLLIP* expression in cancers³⁵⁻³⁷.

Interestingly, we observed hypomethylation of *TLR5* indicating that sex steroid hormones can induce the silencing of the regulators of *TLR5* signals and at the same time activate *TLR5* expression thereby allow

signaling by up regulating different inflammatory molecules in prostate cancer progression. The activation of TLR5 expression by hypomethylation in prostate cancer has been reported by one group³⁸. We also observed hypomethylation of MHC class 1-related chain B (MICB) a ligand of the NKG2D receptor expressed by natural killer cells and activated CD8 (+) T cells, in response to all hormonal treatment, except dexamethasone, which showed the opposite effect. One report by Tang et al.³⁹ demonstrated that MICB is regulated by DNA methylation and that MICB is upregulated following 5-Aza-2'-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor (5-aza-dC) treatment. Other genes that were hypermethylated in all hormone treated cells are the lymphocyte-activation gene 3 (*LAG3*) and the multi paracaspase (*MALT1*) genes, although the biological consequences of hypermethylation of these genes has not been reported in cancer. Many pro-inflammatory cytokines including interleukins (ILs) are frequently claimed to be epigenetically regulated in cancers- DNA methylation has been reported for IL-1B, IL-6 and IL-8 genes in lung cancer⁴⁰. IL12RB2 methylation has been reported in patients with chronic obstructive pulmonary disease and non-small-cell lung cancer (NSCLC)⁴¹. In prostate cancer, the expression level of IL6 has been found to be elevated in both serum and local tissue samples taken from patients with PCa and that IL6 hypomethylation may be a marker for more widespread (possible even genome wide) methylation in PCa⁴². We report hypomethylation of several of these proinflammatory cytokines in our analysis, for instance, *IL1F8*, *IL17C*, *IL13*, *IL12B*, and their receptor *IL4R* are hypomethylated. We have also observed hypomethylation of the leukotriene B4 receptor (*LTB4R*) in all hormone induced treatment (except dexamethasone). Differential methylation levels of *LTB4R* have been observed in other tissues; in a study by Cheong et al.⁴³ they report hypomethylation of *LTB4R* in nasal polyps, whereas another study observed hypermethylation of *LTB4R* in ovarian tumors⁴⁴ suggesting that the methylation status for *LTB4R* gene is tissue specific.

In summary, we have used a PCR approach to simultaneously interrogate the DNA methylation status of 96 genes that are involved in inflammatory and autoimmune signals in response to sex steroid hormone stimulation of prostate cancer cells. Our observations demonstrate the hypomethylation and the concomitant activation of proinflammatory cytokines or the hypermethylation and the concomitant silencing of negative regulators of inflammatory cytokine signals contribute to prostate carcinogenesis. The gene panel array used in our studies allows the correlation of CpG island methylation status with transcriptional status, biological phenotypes, or disease outcomes. Therefore, these results can provide insights into the molecular mechanisms and biological pathways and aid in the discovery and development of biomarkers. In this study, the use of 96 gene panel allowed for the simultaneous analysis of several genes. However, for each time point and concentration an individual 96-well plate is required, as such multiple determinants were not conducted and statistical analysis of significant differences in methylation levels could not be deduced. Therefore additional validation of DNA methylation for each

gene will be needed to rule out variance in the methylation analysis and gene expression. However, our data of methylation and gene expression correlated quite well to suggest little variance in methylation and gene expression level. Despite these limitations, our experiment yielded several interesting and potentially relevant findings of the effect of sex steroid hormones and DNA methylation in prostate cancer that add support to induction of inflammatory genes via hypomethylation may be a potential pathway for prevention. Additional studies are needed to confirm these findings, to screen other cell lines and to further characterize the role and impact of epigenetic changes in sex steroid hormone induced inflammation as well as also look at other potential epigenetic mechanisms affecting inflammation such as histone modification or microRNAs.

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Table: Gene abbreviation and list of primer sequences used in Taqman RT-PCR assays.

Figure legends:

Figure 1: Effect of sex steroid hormones induction on LNCaP cell growth. Briefly, LNCaP cells were seeded on a 6-well plate at 1×10^6 cells per well in complete growth medium. After 24 hours, cultures were replaced with medium containing 2% charcoal-treated FBS to remove steroids, followed by an additional 24 hours in RPMI containing 2% charcoal-treated FBS. Twenty-four hour later, cultures were replaced with The synthetic ligands; R1881 (10⁻¹¹M, 10⁻¹⁰M), testosterone (10⁻⁹M, 10⁻⁸M), 17 β -estradiol (10⁻⁹M, 10⁻⁸ M), progesterone (10⁻⁹M, 10⁻⁸M) and dexamethasone (10⁻⁸M, 10⁻⁷M) and vehicle (EtOH; Ctrl) were each added to cells maintained as above. Cell proliferation was assessed by Coulter counter. The data is represented as the mean of experiments; bars represent standard error of mean. Two way anova test was used to investigate significant differences in fold change. Each hormonal treatment experiment was done at least 3 times.

Figure 2: Clustering analysis of the intensity of promoter methylation of the 96-genes panel in (MeAH-3520E; Human Inflammatory Response & Autoimmunity DNA Methylation PCR Array signature Panel). The LNCaP cells were cultured in the presence or absence of different hormones (0.1 nM R1881; 10nM

Testosterone; 10nM Estradiol; 1mM and 0.1 nM each of R1881 + Estradiol) for 72 hours. Genomic DNA was extracted from LNCaP cells and used in PCR analysis with the Methyl-Profiler DNA Methylation system. Data shows genes which exhibit a difference of 10% in methylation level for at least one hormone treatment versus mock treatment for each gene. Color code and intensity indicate level of methylation (negative values correspond to hypomethylation and positive values correspond to hypermethylation).

Figure 3: The LNCaP cells were treated with or without R1881 (0.1 nM) for 72hours and total RNA extracted and used in RT-PCR with the MAH-001 cytokine array panel. Columns for each gene represent the difference in gene expression between hormones treated and untreated cells. Gene expression was calculated relative to control (mock treated cells and set at 1). Gene expression above 1 indicates increase in gene expression, whereas the values below 1 indicate reduction in gene expression.

Figure 4: The relative mRNA transcript expression levels of CXCL5, LTB4R, CCR1, CCL18 and IL5 were analyzed in prostate cells and tissue samples by RT-PCR and expressed relative to β -actin to correct for variation in the amount of reverse-transcribed RNA. **A.** Heat map analysis of gene expression levels in prostate cell lines; the primary epithelial cell line pNT1A; the androgen dependent cell line LNCaP, and the androgen independent cell lines PC3 and DU145. **B.** Box plot of relative gene expression (shown as log expression on the *Y* axis) in 40 match normal (NI) and prostate cancer (Can) tissues. Unpaired t-test was used to determine statistical significance; P-value; $P < 0.05$ shows significance. **C.** The androgen-dependent prostate cancer cell line, LNCaP was grown in RPMI containing 2% charcoal-treated FBS and R1881 (10^{-10} M) plus 5'-aza-dC (5 μ M) for 96 hours with or without TSA (250 nM) for additional 24 hours. complete growth medium and treated with 5'-aza-2'-deoxycytidine; ($5\mu\text{mol}^{-1}$) or in combination with Trichostatin A (TSA; 250 nmol^{-1}). The gene expression was determined by quantitative reverse-transcriptase-PCR and expressed relative to GAPDH to correct for variation in the amounts of reverse-transcribed RNA. The data are representative of three independent experiments. The fold change in gene expression level was calculated relative to the mock control (R1881 only treated cells) that was set at an arbitrary unit of 1.

Figure 1

Figure 2

Figure 3

Figure 4a

Figure 4b

Fig 4c