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Expression of UDP Glucuronosyltransferases *2B15* and *2B17* is associated with methylation status in prostate cancer cells

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ABSTRACT

Studies have suggested that abrogated expression of detoxification enzymes, UGT2B15 and UGT2B17, are associated with prostate tumour risk and progression. We investigated the role of EGF on the expression of these enzymes since it interacts with signalling pathways to also affect prostate tumour progression and is additionally associated with decreased DNA methylation. The expression of *UGT2B15*, *UGT2B17*, *de novo* methyltransferases, *DNMT3A* and *DNMT3B* was assessed in prostate cancer cells (LNCaP) treated with EGF, an EGFR inhibitor PD16893, and the methyltransferase inhibitor, 5-azacytidine, respectively. The results showed that EGF treatment decreased levels of expression of all four genes and that their expression was reversed by PD16893. Treatment with 5-azacytidine, markedly decreased expression of *UGT2B15* and *UGT2B17* over 85% as well as significantly decreased expression of *DNMT3B*, but not the expression of *DNMT3A*. *DNMT3B* siRNA treated LNCaP cells had decreased expression of *UGT2B15* and *UGT2B17*, while *DNMT3A* siRNA treated cells had only moderately decreased *UGT2B15* expression. Treatment with DNMT methyltransferase inhibitor, RG108, significantly decreased *UGT2B17* expression. Additionally, methylation differences between prostate cancer samples and benign prostate samples from an Illumina 450K Methylation Array study were assessed. The results taken together suggest that hypomethylation of the *UGT2B15* and *UGT2B17* genes contributes to increased risk of prostate cancer and may provide a putative biomarker or epigenetic target for chemotherapeutics. Mechanistic studies are warranted to determine the role of the methylation marks in prostate cancer.

Introduction

In the U.S., cancer is the second leading cause of death and prostate cancer is the second highest occurring cancer in men after lung cancer. The number of new prostate cancer cases for 2020 was estimated to be 191,930[1]. For over 10 years, the DNA sequence variants of the enzymes of the human UDP-glucuronosyltransferases 2B (UGT2B) subfamily have been investigated and shown to be associated with increased risk for prostate cancer [2,3]. The UGT2B subfamily of enzymes are part of the UGT (UGT, EC 2.4.1.17) superfamily of drug-metabolizing enzymes that are bound to the endoplasmic reticulum in hepatic and extrahepatic cells to facilitate the clearance of exogenous and endogenous toxins [4]. The UGT superfamily consists of two main gene subfamilies, *UGT1*, located on chromosome 2 which contains nine unique first exons that are spliced to common exons 2–5; and *UGT2*, located on chromosome 4 which contains nine distinct

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genes. The UGT2 subfamily is further divided into UGT2A and UGT2B[5].

The UGT2B subfamily is comprised of *UGT2B4, UGT2B7, UGT2B11, UGT2B15, UGT2B17,* and *UGT2B28* genes along with six pseudogenes that are interspersed among the genes in the region[6]. Enzymes UGT2B15 and UGT2B17 exhibit substrate specificity for androgens such as testosterone, dihydrotestosterone (DHT), DHT metabolites, androsterone, and 5α-androstane-3α, 17β-diol [7–9]. One of the tissues where the UGT2B15 and UGT2B17 enzymes typically catalyse the clearance of these chemicals is the prostate. Results from a recent study were suggestive of an association of overexpression of *UGT2B17* with biochemical recurrence of prostate cancer in a Veterans' Medical Centre (VA) population including both African American and Caucasian patients [10]. Other lines of evidence showed that *UGT2B15* genes are associated with risk for prostate cancer in African American men [3,11]. These associations suggest that the development of prostate cancer disease has *UGT2B* genetic underpinnings. However, the impact on gene expression remains unclear.

Other lines of evidence suggest that epigenetic alterations may play a role in the regulation of *UGT2B15* and *UGT2B17*. For instance, hyper and hypomethylation changes in the TSS1500 interval of *UGT1A* and *UGT2* genes have been differentially associated with transcription in individuals with non-alcoholic fatty liver disease[12]. On the other hand, DNA hypermethylation and histone hypoacetylation has been shown to abrogate transcriptional regulation of *UGT1A1* in human kidney cells[13]. The investigators in that study further showed that tissue-specific expression of *UGT1A10* was mediated by transcription factors whose efficacy was affected by promoter methylation of that gene[14].

An early study of *UGT2B15* and *UGT2B17* gene expression suggested a regulatory role for epidermal growth factor (EGF)[15]. That study and others showed that *UGT2B17* expression and glucuronidation function was downregulated in prostate cancer cells treated with EGF [16,17]. Aberrant expression resulting from EGF/EGFR signalling has been observed in androgen-independent and metastatic prostate cancer; associated with epithelial–mesenchymal transition and reduced survival rate[18]. This signalling impacts the efficacy of UGT glucuronidation and subsequently its antiproliferative effects on prostate cancer cells in androgen-dependent cancer[19]. Moreover, EGF was later found to alter global DNA methylation in gastric cancer cell lines when used as treatment[20]. In addition, another study suggests that EGF may impact methylation status by modulating DNA methyltransferase activities[21]. In that study, long-term EGF treatment or constitutively active *EGFR* expression increased DNA methyltransferase activity in ovarian cancer cells. These changes are widely believed to be caused by *de novo* methylation by DNA methyltransferases, *DNMT3A* and *DNMT3B*; which target DNA sequences in a complex with DNA methyltransferase, *DNMT3L*, that subsequently becomes associated with histone 3 unmethylated at lysine 4 (H3K4)[22].

The regulation of *UGT* expression by DNA methylation and its impact on *UGT2B15* and *UGT2B17* expression in prostate cancer cells is unknown. Thus, we sought to determine the relationship of EGF/EGFR regulation and DNA methylation on *UGT2B* gene expression in prostate cancer cells. We hypothesized that EGF downregulation of *UGT2B* gene expression in prostate cancer cells is associated with changes in DNA methyltransferase activity. Here, we show that changes in *UGT2B17* and *UGT2B15* expression were altered in the prostate cancer cell line, LNCaP, treated with *DNMT3A* or *DNMT3B* siRNAs. In addition, we present data that shows specific *UGT2B17* and *UGT2B15* CpG sites are associated with prostate cancer as determined by data from the Illumina Infinium HumanMethylation 450K Beadchip array.

Results

EGF downregulated gene expression in prostate cancer cells is reversed by EGFR inhibitor, PD168393

Using RT-PCR, expression of *UGT2B15, UGT2B17, DMNT3A,* and *DNMT3B* was measured in LNCaP cells treated with EGF and the EGFR inhibitor, PD68393 [23]. Treatment with 10 ng/ml EGF decreases *UGT2B15* mRNA expression by nearly 50% when compared to control (p < 0.0001) and that the inhibition is completely reversed by treatment with 10 μM of PD168393 (Figure 1(a)). *UGT2B17* mRNA levels are also reduced by more than 50%
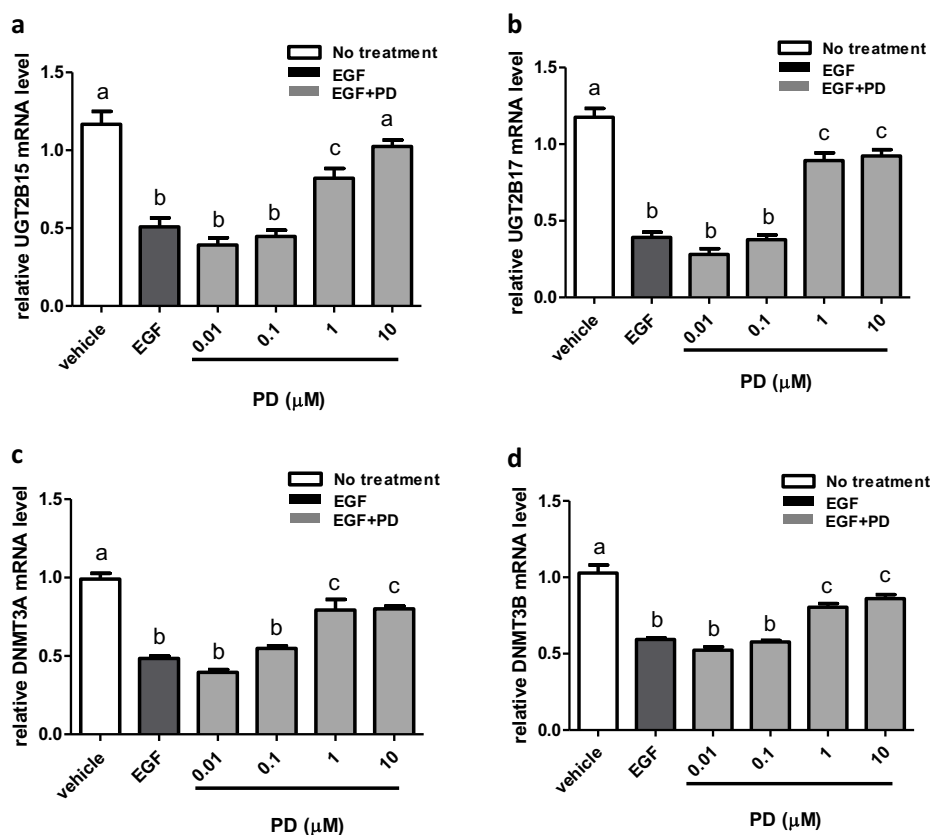
Figure 1. EGF mediated down regulation of *UGT2B15, UGT2B17, DNMT3A and DNMT3B* expression is reversed by EGFR inhibitor, PD168393 (PD). LNCaP cells were treated for 24 h with vehicle (DMSO), EGF and EGF+PD at concentrations indicated in the figure. mRNA was extracted and quantitated using RT-PCR as described in materials and methods. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. The mRNA levels are reported as the mean ± SEM of 3 independent experiments, each performed in triplicate. Means that do not share letters are significantly different from each other. (a) Effect of EGF and PD168393 on *UGT2B15* expression. (b) Effect of EGF and PD168393 on *UGT2B17* expression (c) Effect of EGF and PD168393 on *DNMT3A* expression. (d) Effect of EGF and PD168393 on *DNMT3B* expression. Vehicle = Control.

with EGF treatment ($p < 0.0001$) when compared to the control while treatment with 1 and 10 μM of PD168393 increases mRNA levels significantly when compared to EGF treatment alone ($p < 0.0001$), (Figure 1(b)). Similarly, mRNA levels of *DMNT3A* are decreased by 50% upon EGF treatment when compared to the control while treatment with 1 and 10 μM PD168393 significantly increases mRNA levels when compared to EGF treatment alone ($p < 0.001$), (Figure 1(c)). Finally, EGF treated mRNA levels of *DNMT3B* are decreased approximately by 50% when compared to the control while treatment with 1 and 10 μM PD168393 significantly increases mRNA levels when compared to EGF treatment alone ($p < 0.0001$), (Figure 1(d)).

In summary, these results show that EGF treatment inhibits *UGT2B15, UGT2B17, DMNT3A*, and *DNMT3B* expression which is then reversed by the *EGFR* inhibitor, PD168393.

Demethylation agent, 5-azacytidine, alters gene expression in LNCaP cells in a manner similar to EGF effect

To assess the effect of 5-azacytidine on the expression of *UGT2B15, UGT2B17, DMNT3A*, and *DNMT3B*, cells were treated with 1–10 μM 5-azacytidine and mRNA was measured using RT-PCR. The results show that 5-azacytidine decreases expression of both *UGT2B15* and *UGT2B17* in a dose-response manner (Figure 2(a,b)). At 10 μM, 5-azacytidine inhibited *UGT2B15* and *UGT2B17* expression by 93.2% and 85.4%, respectively ($p < 0.0001$), and inhibited *DNMT3B* expression by 33% ($p < 0.0001$). In contrast, the mRNA levels of *DNMT3A* remained unchanged (Figure 2(c,d)).

Increased expression of both *p53* and *EGFR* have been previously determined in prostate cancer[24]. To demonstrate the gene-specific response
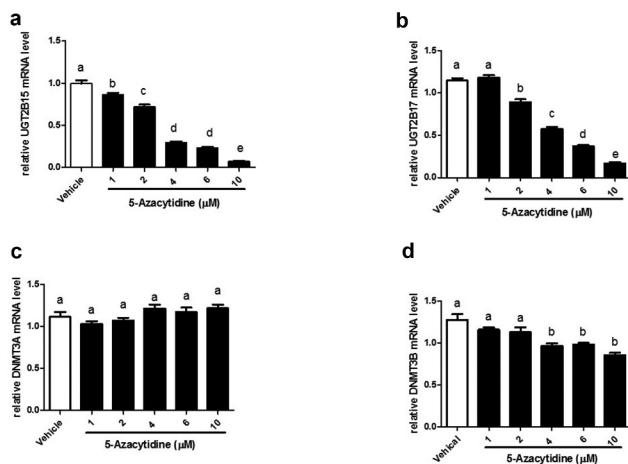
Figure 2. 5-Azacytidine treatment of LNCaP cells downregulates expression of *UGT2B15, UGT2B17* and *DNMT3B* mRNA in a dose-response manner. LNCaP cells were treated with vehicle (DMSO) and increasing amounts (1–10 μM) of 5-Azacytidine for 24 h. mRNA was quantitated and statistically analysed as described in Figure 1 legend. The mRNA levels are reported as the mean ± SEM of 3 independent experiments, each performed in triplicate. Means that do not share letters are significantly different from each other. (a) Effect of 5-Azacytidine on *UGT2B15* expression. (b) Effect of 5-Azacytidine on *UGT2B17* expression. (c) Effect of 5-Azacytidine on *DNMT3A* expression and (d) Effect of 5-Azacytidine on *DNMT3B* expression. Vehicle = Control.

of *UGT2B* and *DNMT3* genes to 5-azacytidine, the impact of treatment with 10 ng/ml EGF, its inhibitor, and 5-azacytdine on *p53* expression in LNCaP cells was determined (Figure S1). The results show that treatment with 1–10 μM 5-azacytidine increases *p53* gene expression in a dose-response manner. At 10 μM, 5-azacytidine increased *p53* gene expression (by 46.7%, p < 0.0001), (Figure S1A). Treatment with 10 ng/ml EGF significantly increases levels of *p53* mRNA which is then reversed by treatment with EGFR inhibitor, PD168393 (Figure S1B).

In summary, these results show that the demethylation agent, 5-azacytidine decreases *UGT2B15, UGT2B17,* and *DNMT3B* expression in a gene-specific manner that mimics the EGF effect. In contrast 5-azacytidine and EGF increases *p53* gene expression.

DNMT3A and DNMT3B silencing downregulates gene expression in LNCaP cells

Because treatment with 5-azacytidine inhibited *UGT2B15, UGT2B17* and *DNMT3B* expression,

we used *DNMT3A* or *DNMT3B* siRNA to confirm that DNMTs drive this effect.

Transfection with *DNMT3B* siRNA decreased *DNMT3B* expression by 72% when compared to cells transfected with non-targeting control (negative control) siRNAs (p < 0.0001) (Figure 3(a)). *DNMT3B* silencing significantly downregulated *UGT2B17* and *UGT2B15* expression by 45% and 47% (p < 0.0001), respectively, when compared to negative control (Figure 3(b,c)). Unexpectedly, however, *p53* mRNA levels were also significantly decreased by 40% (p < 0.0001) (Figure 3(d)) after having observed increased expression when hypomethylated by 5-azacytidine.

Transfection with *DNMT3A* siRNA reduced *DNMT3A* by 60% (p < 0.0001) when compared to cells transfected with nonsense siRNAs (Figure 3(e)). *DNMT3A* silencing slightly decreased expression of *UGT2B17* and *p53* genes (Figure 3(f,g)), but this reduction was not significant. However, levels of *UGT2B15* mRNA were significantly decreased by 16% (p < 0.02) (Figure 3(h)) when compared to non-sense controls.

In summary, *DNMT3B* silencing in prostate cancer cells effectively and significantly decreases expression of *UGT2B15, UGT2B17,* and *p53*; whereas *DNMT3A* silencing significantly reduces only *UGT2B15* expression.

Methyltransferase inhibitor, RG108, alters UGT2B17 expression in LNCaP cells

To assess the effect of directly inhibiting DNA methyltransferase activity, LNCaP cells were treated with methyltransferase inhibitor, RG108 [25]. This inhibitor was identified through a screen using methyltransferase, *DNMT1*; however, its highly conserved catalytic domains are shared with methyltransferases, *DNMT3A* and *DNMT3B*. The results show that mRNA levels were unchanged between vehicle and RG108 treatment for *DNMT3A, DNMT3B,* and *p53* (Figure 4(a,b,d), respectively). Notably, the expression of *UGT2B17* was decreased by 32% (p < 0.0001) (Figure 4(c)).

In summary, the inhibition of methyltransferase activity with RG108 unlike 5-azacytidine only altered *UGT2B17* gene expression.
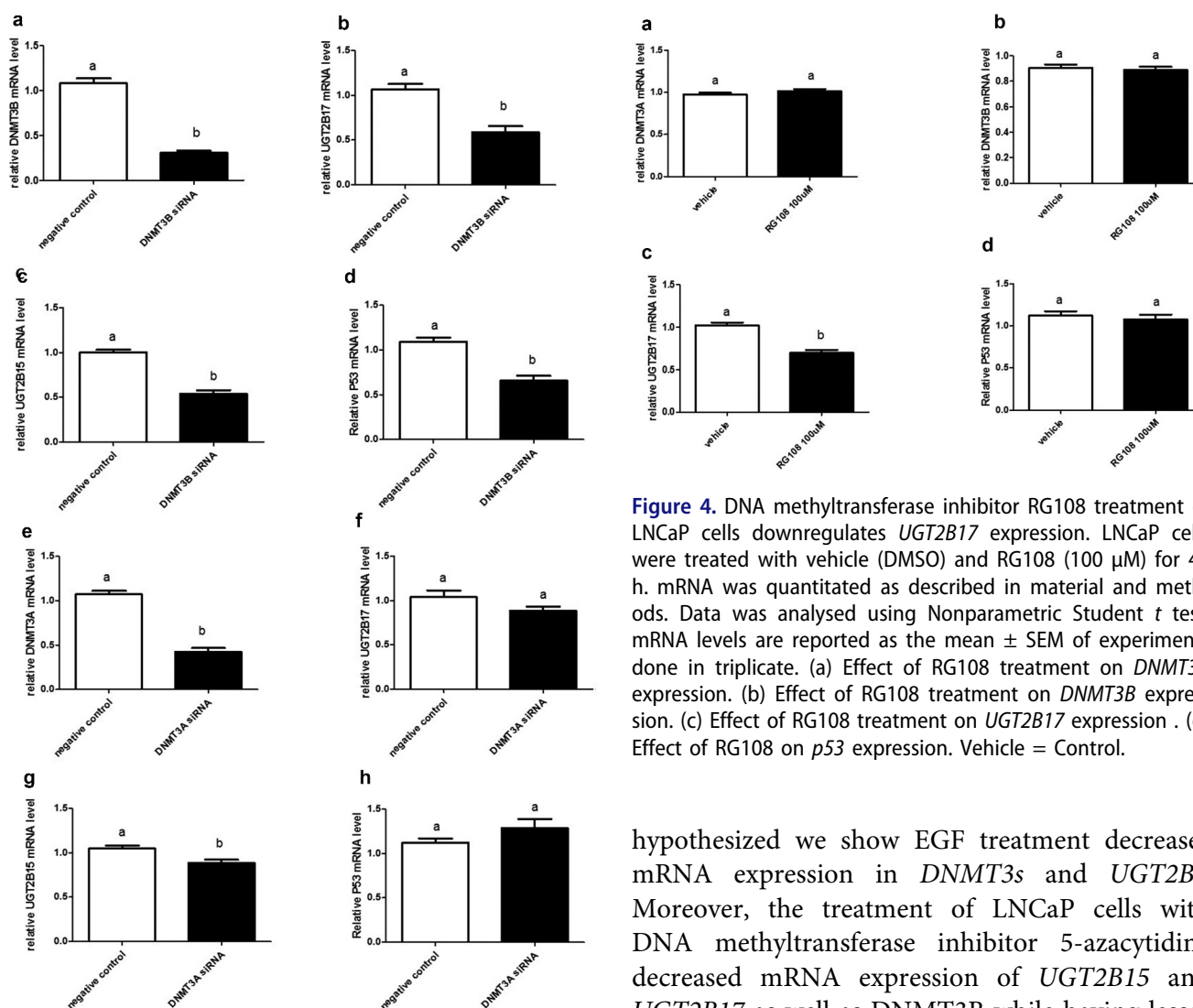
Figure 4. DNA methyltransferase inhibitor RG108 treatment of LNCaP cells downregulates *UGT2B17* expression. LNCaP cells were treated with vehicle (DMSO) and RG108 (100 μM) for 48 h. mRNA was quantitated as described in material and methods. Data was analysed using Nonparametric Student *t* test. mRNA levels are reported as the mean ± SEM of experiments done in triplicate. (a) Effect of RG108 treatment on *DNMT3A* expression. (b) Effect of RG108 treatment on *DNMT3B* expression. (c) Effect of RG108 treatment on *UGT2B17* expression . (d) Effect of RG108 on *p53* expression. Vehicle = Control.

Figure 3. *DNMT3B* and *DNMT3A* siRNA knockdown in LNCaP cells showing knockdown expression of *UGT2B17, UGT2B15*, and *P53*. The mRNA was quantitated and statistically analysed as described in Figure 1 legend. The mRNA levels are reported as the mean ± SEM of 3 independent experiments, each performed in triplicate. Means that do not share letters are significantly different from each other. (a) Knockdown expression of *DNMT3B*; (b) *UGT2B17* in *DNMT3B* knockdown LNCaP cells; (c) decreased *UGT2B15* in *DNMT3B* knockdown LNCaP cells at p < 0.0001; and (d) decreased *p53* in *DNMT3B* knockdown LNCaP cells at p < 0.0001. *DNMT3A* siRNA knockdown in LNCaP cells showing (e) knockdown expression of *DNMT3A*; (f) decreased *UGT2B17* in *DNMT3A* knockdown LNCaP cells at p < 0.0001; (g) decreased *UGT2B15* in *DNMT3A* knockdown LNCaP cells at p < 0.02; and (h) *p53* in *DNMT3A* knockdown LNCaP cells. Negative control = negative control siRNA; *DNMT3A* or *DNMT3B* siRNA = NeoFX Transfection Agent 0.8 ul (30 nM) siRNA.

Discussion

Previous studies have suggested an association between EGF modulated gene expression in cancer cells and DNA methylation status. As hypothesized we show EGF treatment decreases mRNA expression in *DNMT3s* and *UGT2Bs*. Moreover, the treatment of LNCaP cells with DNA methyltransferase inhibitor 5-azacytidine decreased mRNA expression of *UGT2B15* and *UGT2B17* as well as DNMT3B while having lesser effect on *DNMT3A* mRNA expression. While the decrease in *DNMT3B* expression was moderate in LNCaP cells treated with 5-azacytidine when compared to that of *UGT2B15* and *UGT2B17*, the decrease in global methyl acceptor activity in the presence of EGF observed in Figure S2 suggests a role for methyltransferases. The results from the silencing of *DNMT3B* suggest an unknown interaction between the methyltransferase in decreasing *UGT2B* gene expression since *p53* expression was decreased by *DNMT3B* siRNA treatment but had the expected increase in expression under conditions created by 5-azacytidine. Results from a study of 73 curated clinically annotated fresh-frozen prostate cancer tissues and 63 benign prostate tissues using the Illumina Infinium HumanMethylation 450K Beadchip array showed significant differences in methylation in the regulatory region of *UGT2B15* and *UGT2B17* that were

Table 1. Analysis of study GSE76938 of 73 prostate cancers vs. 63 benign tissue with Illumina HumanMethylation 450 K Beadship array.

probeID	BETA*	SE	P_VAL	padj	Gene Name(s)	Location	Reference
cg20840634	−0.1164	0.0165	1.9285E-12	1.2535E-11	UGT2B15;UGT2B17	TSS1500	Oeser et al 2018
cg22161115	−0.0676	0.0124	4.5561E-08	1.6923E-07	UGT2B15	1stExon	Oeser et al 2018
cg21846159	0.0317	0.0099	0.0014	0.0042	UGT2B15	Body	Oeser et al 2018
cg10632656	0.0367	0.0152	0.0159	0.0344	UGT2B15;UGT2B17	Intergenic	Oeser et al 2018

*Negative BETA value is Hypomethylation and positive is Hypermethylation.

indicative of hypomethylation (Table 1; cg20840634; 11.64% (p = 1.9 x 10 $^{-12}$))[26]. Taken together these results suggest that DNA methyltransferases play a role in *UGT2B* gene regulation (Figure 5).

Research nearly twenty years ago first demonstrated that LNCaP cells treated with EGF had decreased expression of *UGT2B17* mRNA but not *UGT2B15* [15–17]. The results in this study showed that the decrease in expression was observed in both *UGT2B15* and *UGT2B17* when measured with gene-specific custom PCR primers [27]. Reversal of the downregulation by *EGFR* inhibitor, PD168393, demonstrates that EGF acts through that receptor in prostate cancer cells to decrease *UGT2B15* and *UGT2B17* expression. The inhibitor has been used in synergy with other chemotherapeutic drugs in prostate cancer patients to abrogate the proliferative effect that has been observed because of *EGFR* activity in cancers [28–30]. In addition, reduced levels of *UGT2B15* and *UGT2B17* from *UGT2B15/B17* siRNA treated cells resulted in lower levels of glucuronidation of *UGT2B15/B17* androgen metabolites leading to increased levels of dihydrotestosterone (DHT)[31]. The inverse relationship between *UGT2B15/B17* expression and levels of DHT may be linked to activated androgen receptor and EGFR interactions associated with progression to castration-resistant prostate cancer (CRPC) or resistance to androgen-derived therapies [32,33]. Thus, decreased expression of *UGT2B15* and *UGT2B17* may be markers for the development of CRPC.

To date, only two studies have shown an association between EGF/EGFR activation and DNA methylation. In one study, 10 nM of EGF was shown to induce the demethylation of DNA in treated gastric cancer cell lines [20] while in another study by Samudio-Ruiz and Hudson [21], EGF treatment of ovarian cancer cells significantly increased DNMT activity and subsequently global DNA methylation. In that same study, the treatment of prostate cancer cells with the same concentration of EGF resulted in decreased global methyltransferase activity consistent with decreased expression of *UGT2B15, UGT2B17, DNMT3A,* and *DNMT3B* observed in prostate cancer cells in our study. Only one study has shown a link between methylation at the *UGT2B15* locus and gene expression[34]. In that study, differences in levels of methylation were found between Asians and Caucasians. Other studies focused on regulation of expression of other enzymes in the *UGT1* and *UGT2* subfamilies by methylation. [12,35] Because there is evidence that EGF increases DNMT methyltransferase activity in ovarian cancer cells, it is highly likely that expression of *DNMT3A* and *DNMT3B* is affected by EGF[21]. That study measured global methyltransferase activity and did not distinguish between *DNMT1, DNMT3A,* or *DNMT3B*. Our study represents the first measurement of specific *DNMT3A/3B* expression in prostate cancer cells treated with EGF and 5-azacytidine. In our study, the silencing of the *DNMT3B* gene demonstrated decreased expression of
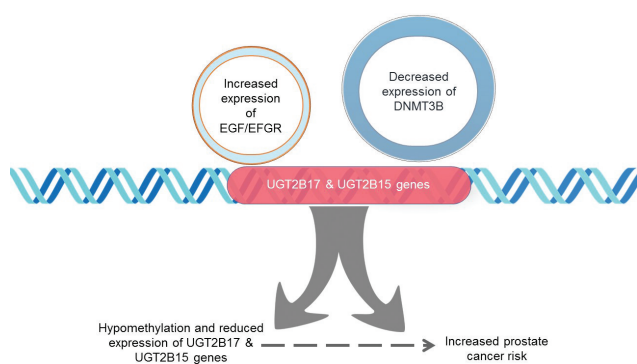

Figure 5. Downregulation of *UGT2B17* and *UGT2B15* gene expression induced by increased EGFR activity and decreased *DNMT3B* expression. Specific CpG dinucleotides within UGT2B regulatory regions are hypomethylated at the proximal promoter and coding region and are associated with prostate cancer risk. EGF/EGFR signalling may mediate the downregulation of DNMT3B methyltransferase activity resulting in decreased expression of *UGTB217* and *UGT2B15* genes from the hypomethylation of regulatory CpG dinucleotides in the promotor and first exon.

UGT2B15 and *UGT2B17* in a hypomethylated cellular environment. In addition, *DNMT3B* and not *DNMT3A* expression in prostate cancer cells was shown to be affected by treatment with a plant-derived carbazole alkaloid, which resulted in the restoration of expression a tumour suppressor gene [36]. These data suggest that *DNMT3A* and *DNMT3B* methyltransferases may have differential roles in regulation of *UGT2B15* and *UGT2B17* expression even though they have very similar responses to EGF treatment. In a study, bicalutamide, used in antiandrogenic therapy, increased the expression of *DNMT3B* but not *DNMT3A* in 22Rv1 cells suggestive of a relationship of DNMT mediated hypermethylation and prostate cancer progression to the castration-resistant phenotype [37]. In that same study that expression was attenuated by treatment with 5-azacytidine. These results are consistent with the observations from another study that showed increased expression of *UGT2B15* and *UGT2B17* in LNCaP cells treated with bicalutamide[33].

Several studies have examined epigenetic mechanisms of gene regulation of UGT enzymes including the methylation of distinct CpG sites [12–14,38-43]. The current literature indicates that only one study has examined the regulation of *UGT2B15* and *UGT2B17* by site-specific DNA methylation in human normal hepatic tissue[34]. In that study, *UGT2B15* was found to be differentially methylated at four CpG loci, cg09189601, cg07973162, cg10632656, and cg07952421, in hepatic samples from Caucasians and Asians. Site cg20840634 (Table 1) was hypomethylated in the transcriptional start site regions of *UGT2B15* and *UGT2B17* in prostate cancer tissue when compared to normal tissue. However, that same loci showed no difference in methylation patterns in hepatic tissue[34]. Further, loci cg22161115, located in the first exon of *UGT2B15*, was also shown to be associated with hypomethylation in prostate cancer tissue when compared to normal tissue but not showing differential methylation in hepatic tissue. These observations are evidence that methylation patterns may differ by gene location and tissue specificity and that specific sites may be an important mechanism in *DMNT3A/3B* mediated gene regulation of *UGT2B15* and *UGT2B17*. While changes in global methylation have been observed in EGF treated gastric cancer cells [20], specific CpG dinucleotides may be responsible for downregulation of *UGT2B15* and *UGT2B17* genes in prostate cancer. Notably, two CpG dinucleotide loci associated with hypermethylation of prostate cancer tissue were located in the body and intergenetic region of *UGT2B15* and *UGT2B15/UGT2B17*, respectively (Table 1).

Future studies that implement more sensitive, genome-wide methods will be needed to determine the relationship between *UGT2B* region methylation and prostate cancer progression including to CRPC to establish the biomarker potential of such patterns. Such approaches have been explored with other genes associated with prostate cancer risk and progression[44].

Materials and methods

Cell culture

LNCaP cells were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI (Gibco, ThermoFisher Scientific, USA), 1% Penicillin-Streptomycin (Gibco), and 10% Heat-Inactivated Foetal Bovine Serum (FBS) (Gibco). Cells were grown in 25 or 75 cm^2 flask (Corning) for siRNA experiments and protein isolation for Western Blots, respectively. Cells were grown to 80% confluency, trypsinized (0.25% Trypsin-EDTA, Gibco ThermoFisher Scientific, USA), collected in a 15-mL tube (Fisher Scientific), and centrifuged (125 x g) for 5 min. The cell pellet was collected and reconstituted in antibiotic-free RPMI supplemented with 2% Charcoal-stripped FBS. Cells were seeded (72 h) in a 96-well plate (Corning, Tweksbury, MA) at 20,000 cells/well, in triplicates, for 24 hr treatments.

Cell culture treatments

For EGF (Gibco, ThermoFisher Scientific, USA) treatment, 100 μl of complete RPMI 1640 media (10% charcoal stripped serum, 1% penicillin/streptomycin, 1% sodium bicarbonate) was added to wells in a 96 well-dish and pre-incubated at 37°C to adjust for pH and temperature. Cells were then added to each well at 200 cells/ μl in a 50 ul volume then incubated at 37°C and 5% CO_2 for 72 hr. The media in the wells was replaced with fresh complete RPMI 1640 media containing 10 ng/ml EGF and 0.01, 0.1,1, and 10 ng/ml EGFR inhibitor. In treatments with PD168393, (Calbiochem Corp., San Diego, CA) DMSO was

used as vehicle for PD168393 and the control consisted of a DMSO vehicle control. For samples with dual treatments of EGF and PD168393, the controls consisted of the DMSO vehicle control. For cells treated with 5-azacytidine (MP Biomedicals, Santa Ana, CA) the pre-incubation procedure was followed but the media in the wells was replaced with fresh complete RPMI 1640 media containing 1, 2, 4, 6 and 10 μM 5-azacytidine. DMSO vehicle control was included in control and 5-azacytidine treated cells. These treatments were incubated for 24 h. For cells treated with 100 μM DNA methyltransferase inhibitor, RG108 [45], DMSO vehicle control was included in control and RG108 treated cells. The experiments were performed in triplicate. All treatments were incubated for 48 hr at standard culture conditions.

Methyltransferase activity measurement

The methyltransferase activity was determined by use of a commercially prepared kit from Fermentas (Fermentas-ThermoFisher Scientific). LNCaP cells were treated with EGF and PD168393 as described above in Cell Culture Treatments in 75 ml culture flasks. The cells were incubated 48 hours and nuclear protein isolated quantitated using Pierce protein isolation kit (Thermo Scientific). Universal DNMT substrates are stably coated onto microplate wells and the activity in nuclear extracts transfer methyl groups to cytosine from Adomet to methylate DNA substrate. The wells are sequentially incubated with different antibodies, washed and a colour developing solution added developed and absorbance measured at 450 nm.

siRNA transfection

Short interfering RNAs (siRNA) specific for human Deoxyribonucleic Acid (DNA) (cytosine-5-) methyltransferase 3 alpha (DNMT3A) (s200426), Deoxyribonucleic Acid (DNA) (cytosine-5-) methyltransferase 3 beta (DNMT3B) (s4223) and Negative Control siRNA were purchased from Applied Biosystems (Foster City, CA). siPORT™ NeoFX™ Transfection Agent (Ambion) and siRNA were individually diluted in Opti-Mem I medium (Gibco), incubated for 10 mins at room temperature (RT), then combined 1:1 to form a siRNA/siPORT™ NeoFX™ complex. The final concentration of siRNA is 30 nM. This complex was incubated for

10 min at RT and added to the cells which were incubated under standard cell culture conditions for 48 hr.

Quantitative real-time PCR

Expression was measured in siRNA treated cells after 48 hr using quantitative-RT PCR (Q-RTPCR) using TaqMan® Gene Expression Cells-to-CT™ (Thermo Fisher-Applied Biosystems, AM1728, Foster City, CA) technology was used to lyse the cells and prepare cDNA. Briefly, the cells were washed with cold 1X Phosphate Buffered Solution (PBS). Next, 50 μl of lysis solution is added, mixed five times and incubated 5 min at room temperature. After 5 μl of stop solution is added and mixed five times the solution is incubated for 2 min at room temperature. Reverse transcription was accomplished by incubating ten microlitres of crude cell lysate with RT Master Mix and running samples in RT thermal cycler program to convert RNA to cDNA. Standard PCR was accomplished using TaqMan PCR Master Mix and 5–10 μl cDNA template using commercially available primers and 7900HT Fast Real-Time PCR Systems with 96 well block (ThermoFisher-Applied Biosystems, AM1728, Foster City, CA). Commercial primers used as follows: TaqMan® Gene Expression Assays™ for DNMT3B (Assay ID. Hs00171876_m1) and DNMT3A (Assay ID. Hs00602456_m1) were purchased from Applied Biosystems (ThermoFisher-Applied Biosystems, AM1728, Foster City, CA). The DNMT3B assay includes six Interrogated Reference Sequences: **NM_001204055.1, NM_001207056.1, NM_0068 92.3, NM_175848.1, NM_175849.1 and NM_1758 50.2**. The DNMT3A assay includes four Interrogated Reference Sequences: **NM_001320892.1, NM_022 552.4, NM_175629.2 and NM_175630.1**. Knock down percentage was quantified by using the comparative $\Delta\Delta C_t$ with 18S as an internal control. Relative abundance of subsequent genes, UDP glucuronosyltransferase family 2 member B17 (UGT2B17) (Assay ID. Hs00854486_sH), UGT2B15 (Assay ID. EX25) and Tumour Protein P53 (TP53) (Assay ID. Hs00153349_m1) were also analysed.

Statistical analysis

Statistical calculations were performed using Prism Version 5.0 (GraphPad Software, Inc., San Diego,

CA). For RT-PCR experiments, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used to determine statistical differences among controls and treatments (EGF, PD168393, RG108 and 5-azacytidine). Data are reported as the mean ± SEM of three independent experiments, each performed in triplicate. Nonparametric Student *t* test was performed to analyse statistical differences between siRNA transfected groups, and comparison of subsequent gene expression. Data are reported as the mean ± SEM of experiments done in triplicate. $p < 0.05$ was considered a significant difference.

GEO analysis

Regularized logistic regression was performed (rlm), and betas determined for ~ Tumour/Normal, between tumour and normal samples for UGT genes from study GSE76938 which examined 73 clinically annotated fresh-frozen prostate tissues and 63 benign prostate tissues using the Illumina Infinium HumanMethylation 450K Beadchip array (Kirby et al., 2017 BMC Cancer April 17;17(1):273. PMID: 28,412,973). Negative BETA values represent hypomethylation and positive represents hypermethylation. The significance is based on multiple-corrected p-value using Benjamine Hockereg multiple correction (padj).

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