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Research Article

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Anti-proliferation and migration efficacy via inhibition of an androgen synthesis key enzyme in castration-resistant prostate cancer

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ABSTRACT

After evaluating by gene differential expression microarray we found that UGT2B15 is highly expressed in castration resistant prostate cancer cell lines. We supposed that docetaxel in combination with UGT2B15 down regulation may have a synergistic effect on tumor cells proliferation and migration. This study aimed to investigate the effects of UGT2B15 silencing on the sensitivity of prostate cancer cells to docetaxel treatment. After transfected with UGT2B15-targeted shRNAs and treated with different concentrations of docetaxel, proliferation and migration of DU145 cells were examined by enumeration in a haemocytometer and wound healing assay. Expression of UGT2B15 protein in DU145 cells was detected by western blot. Results demonstrated that silencing of UGT2B15 promoted docetaxel induced cell growth inhibition and inhibited cell migration in CRPC. Results indicate that UGT2B15 may be used as a new promising diagnostic biomarker and a potential anticancer therapeutic target for CRPC.

Key words: UGT2B15-shRNA, down-regulation, CRPC, DU145

INTRODUCTION

Androgen deprivation therapy remains the single most effective treatment for the initial therapy of advanced prostate cancer [1], but is uniformly marked by progression to castration-resistant prostate cancer (CRPC) [2]. Docetaxel (DTX) is the first-line chemotherapeutic option for symptomatic CRPC patients who are candidates for chemotherapy [3], which enhances the overall response, clinical remission of the prostate cancer patients [4]. However, cytotoxicities especially hematopoietic side-effects are significant and inevitable progression occurs after DTX treatment [5, 6]. Therefore, it is important to identify better or alternative therapeutic strategies to enhance the sensitivity of docetaxel-based chemotherapy.

Study indicated that CRPC remains androgen dependent and androgen synthesis inhibitor (ASI) can enhance the sensitivity of chemotherapy in CRPC patients [7]. Recurrent PCa was previously referred to as androgen independent prostate cancer due to the tumor growth continued in the presence of castrate levels of circulating androgens. However, studies found that the tumor proliferation occurs as a result of the reactivation of the androgen receptor (AR) within the tumor despite the low levels of circulating androgens [8, 9]. AR usually become aberrant due to selective stress of pharmacological inhibitors of AR and CRPC often occurs due to aberrant reactivation of AR. CRPC arising maybe dependent on intratumoral androgen biosynthesis which is due to elevated expression of enzymes responsible for androgen production [10]. Reagents that target the androgen axis by inhibiting androgen biosynthesis and or AR signaling are potential candidates for CRPC treatment and are currently being pursued aggressively [11, 12].

The UDP-glucuronosyltransferase *(*UGT*)* genes code for enzymes that convert a diverse group of xenobiotic and endobiotic substances into lipophilic compounds, facilitating clearance from the body as part of the phase II liver detoxification system. It plays a pivotal role in androgen biosynthesis and in the pre-receptor regulation of AR action within the prostate. Several studies indicate that UGT2B15 is overexpressed in prostate cancer and that expression increases with the progression of the disease [13, 14]. These enzymes exhibit specificity for androgen metabolites such as testosterone, dihydrotestosterone (DHT), androsterone (ADT), and androstane-3α,17β-diol (3β-diol) in prostate tissue and cell lines. Consequently, UGT2B15 expression and activity has been implicated in the development of CRPC, making it a rational target [15]. Therefore, we explored the therapeutic potential of inhibiting UGT2B15 signaling in the treatment of CRPC especially in combination with chemotherapy.

In this study, we used shRNA-mediated selective inhibition of UGT2B15 to investigate the efficacy of docetaxel sensitivity in DU145 cells. Furthermore, the effect of UGT2B15 silencing in combination with docetaxel treatment on cancer cell migration was also examined.

EXPERIMENTAL SECTION

Cells culture and drug

DU145 prostate cancer cells were provided kindly by Prof. Peter S Nelson (Fred Hutchinson Cancer Research Center) as a gift. Cells were grown at 37 ℃ under 5% CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and 1% penicillin streptomycin (Gibco). Confluent cells were passaged with trypsin-EDTA (0.05% trypsin and 0.53 mM tetrasodium EDTA) prepared to be transfected by UGT2B15-targeted shRNA. Docetaxel was purchased from R&D Systems, Sigma–Aldrich and Enzo Life Science.

RNA interference and transfection

Small interfering RNAs (shRNAs) were purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). UGT2B15-targeted shRNA sequence was 5′-GGU GAG GAA CUU UCA CCA A-3′ and a scrambled oligonucleotide sequence were chemically synthesized used as a negative control (unrelated shRNA). DU145 cells were seeded into 6 well plates (1×10^6 cells per well) and grown to 70% confluency, replenished in serum-free Optimem 1, then transfected for 4h with a shRNA oligonucleotide pool targeting UGT2B15, using oligofectamine according to manufacturer's instructions. A non-targeting oligonucleotide pool was used at the same concentration as a transfection control. The expression of UGT2B15 was assayed by western blotting 72 h after transfection.

Western blot analysis

Preparation of total cell lysate and the procedures for western blot analyses were performed essentially as described by Day et al [16]. Protein samples were separated on 10% polyacrylamide resolving gels with the buffer system and transferred onto nitrocellulose membranes for 2h at 250 mA. Protein binding sites on the nitrocellulose were blocked for 1h at 25 ℃ in 5% (w/v) Marvel/PBS/3% (v/v) Tween-20 (PBST), then incubated overnight at 4 ℃ with UGT2B15 monoclonal antibodies (1:1000 dilution; Invitrogen, California, USA). The membrane was washed 3×10 min in TBST and probed with horseradish peroxidise-conjugated secondary antibodies (Amersham Life Sciences, Buckinghamshire, UK) for 1h at 25 ℃. Following 3×10 min washes in PBST, bands were detected using enhanced chemiluminescence (ECL+ reagents, Amersham). Densitometric quantification of band intensities was performed using Kodak one-dimensional image analysis software.

Cell proliferation and viability assay

DU145 prostate cancer cells were seeded in 96-well plates in quintuplicate with Dulbecco's basal medium plus 10% fetal bovine serum and maintained in culture for 24 h. After transfected with UGT2B15-targeted shRNA and the negative control the cells were treated with DTX (5, 10, 20, 50 or 100 nM) for 24h, 48h and 72h, respectively. At the end of incubations, cell medium was removed and 100 μL/well of MTT solution (0.5 mg/mL in PBS) were added. After incubation at 37 ℃, 5% CO2 for 4h, the supernatants were removed carefully, and 150 μl of DMSO was added to each well. Absorbance was measured at 450 nm in a Microplate Reader (Bio-Rad 680). Analysis of the obtained results was done using GraphPad Prism 4 computer program to evaluate cell proliferation rate and cytostatic rate. A hemacytometer count and microscopic evaluation of the viable cells were used as the alternative method. DTX untreated cells were used as controls.

Wound healing assay

The scratch assay was performed to assess the influence of DTX on shRNA transfected prostate cancer cell motility. DU145 cells were seeded in a high density at 6-well plates and grown for 24 h, transfected with the shRNA against UGT2B15 and non-targeting oligonucleotide (unrelated shRNA) as a control for 4 hrs then treated with 20 mM docetaxel. Then the monolayer cells were physically wounded by scratching the surface with a pipette tip (1000 μl) as uniformly and straight as possible. The images of cells invading the scratch were captured at indicated time points

(0, 4, 6, 8 and 24 hrs) using phase contrast microscopy (IX 70 Olympus Optical Co., Germany). The pictures were evaluated by measuring the difference in the area of the wounds with a Leica image analysis system (Leica, Mannheim, Germany) and migration rate expressed as percentage of scratch closure was calculated as follows: % of scratch closure= a-b/a, where (a) is a distance between edges of the wound, and (b) is the distance which remained cell-free during cell migration to close the wound. The experiments were repeated in triplicate wells at least two times.

Statistical analysis

The data from the experiments were analyzed using the SPSS 18.0 software and expressed in the form of the mean ± SD. The two- tailed Student's t-test was used to analyze the differences in the experiments. Results were considered significant at $p<0.05$ for all analyses.

RESULTS

Effect of UGT2B15-targeted shRNA on expression of UGT2B15 gene in DU145 cells

To determine the efficiency of the downregulation of UGT2B15-targeted shRNA in DU145 cells, three small interfering RNAs and non-targeting oligonucleotide pool (unrelated shRNA) were used to transfected into DU145 cells in accordance with the manufacturer's recommendations. Protein expression levels were determ- ined quantitatively by western blot analyses. As demonstrated in Figure 1, comparing to untreated control DU145 cells, the UGT2B15-targeted shRNA-1, -2 and -3 significantly decreased UGT2B15 protein levels to 42.27 ± 2.58%, 62.98 ± 3.75% and 67.65 ± 3.18% at 72 h post-transfection respectively, whereas, UGT2B15 protein was strongly expressed in non-transfected and transfected with shRNA with non-targeting sequence. The results demonstrated that UGT2B15 expression was significantly inhibited after infection with specific UGT2B15-targeted shRNA ($p<0.01$ compared with control groups). UGT2B15-targeted shRNA-1 was chosen to assess the impact of UGT2B15 expression on the proliferation and viability of PC3 cells through MTT assay or counting method.

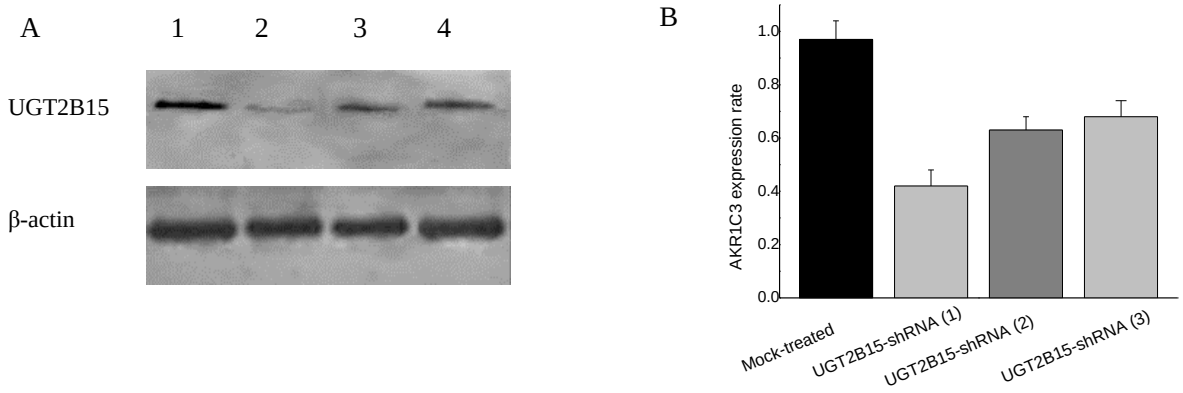

Figure 1. (A) Protein expression of UGT2B15 was detected by western blotting. (B) **Densitometric analysis was performed using Kodak one-dimensional image analysis software. Lane 1, shRNA/non-targeting oligonucleotide (unrelated shRNA); and lane 2,3,4, UGT2B15-targeted shRNA-1, -2 and -3 respectively. ($^{**}P < 0.01$)**

Downregulation of UGT2B15 expression increases chemosensitivity

To examine whether downregulation of UGT2B15 could sensitize prostate cancer cells to docetaxel, a combination of docetaxel and UGT2B15-targeted shRNA was used to treat DU145 cells, and MTT assays were performed to measure the proliferation of the tumor cells. DU145 cells were transiently transfected with UGT2B15-targeted shRNA or control shRNA for 48 hours and treated with docetaxel for an additional 24 hours at different concentrations including 0, 5, 10, 20, 50 and 100 nM. Results revealed that silencing UGT2B15 prior to docetaxel treatment significantly potentiated the inhibitory effects of docetaxel on cell proliferation, compared to the non-targenting oligonucleotide or docetaxel alone treatment groups. Treatment with DTX at a concentration of 10 uM caused a reduction of viable cells to 76% and addition of 20 uM could reduce significantly the viable cell number to 58% (Fig. 2A). However, an additive effect was observed for DU145 cells when combining DTX at 10 nM and UGT2B15-targeted shRNA treatment and the cell viability markedly reduced to 34% compared with the bicombination of DTX and unrelated shRNA treatment (Figure 2B). At lower DTX concentrations the effects from this drug were negligible and mainly from UGT2B15-targeted shRNA inhibition.

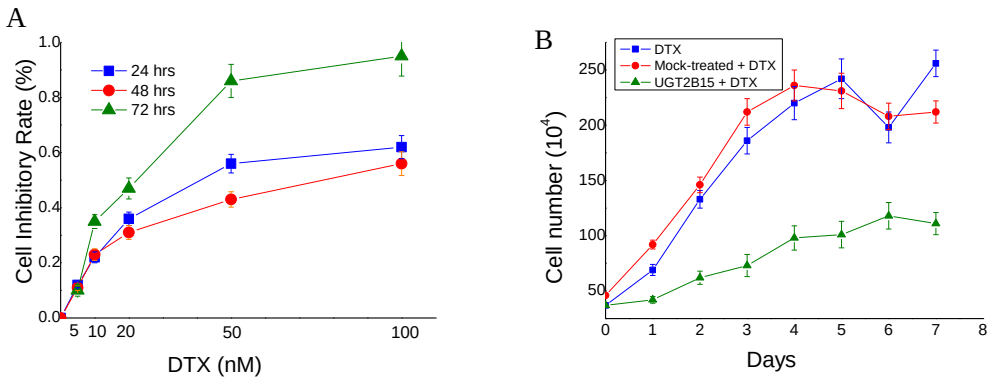

Figure 2. Susceptibility of DU145 cells to different concentrations of DTX or 10nM DTX after silencing UGT2B15. A. Cells were treated with increasing concentrations of DTX in combination UGT2B15-targeted shRNA treatment and cell viability was assayed using MTT assays. B. Cells were transfected with UGT2B15-targeted shRNA or non-targeting sequence of shRNA, then treated with 10 nM docetaxel and viable cells were counted using a hemacytometer at different time point
*Values represent mean ±SD (*n = *4).*

Effect of DTX, UGT2B15-targeted shRNA alone and the combined treatment on the migration of the DU145 cells

To detect the effect of docetaxel in combination with UGT2B15-targeted shRNA downregulation on the migration of DU145 cells, a wound healing assay was performed following 24 h after DTX treatment. Results showed that both docetaxel and UGT2B15-targeted shRNA treatment alone have the inhibitory effect on cells migration. However, cells treated with DTX in combination with UGT2B15-targeted shRNA migrated more slowly than those treated with the drug or UGT2B15-targeted shRNA alone. Compared with the control (Fig.3), the relative migrated distance of the cells in the DTX, UGT2B15-targeted shRNA and DTX/UGT2B15-targeted shRNA, respectively were 72.3±8.11, 78.2±7.13, 41.4±6.24 ($p<0.05$). The cells incubated in the DTX only migrated across an area that was markedly larger than that of the cells in the combinational treatment of DTX and UGT2B15-targeted-shRNA, indicating that DTX in combination with UGT2B15 downregulation significantly inhibited the migration of the DU145 cells.

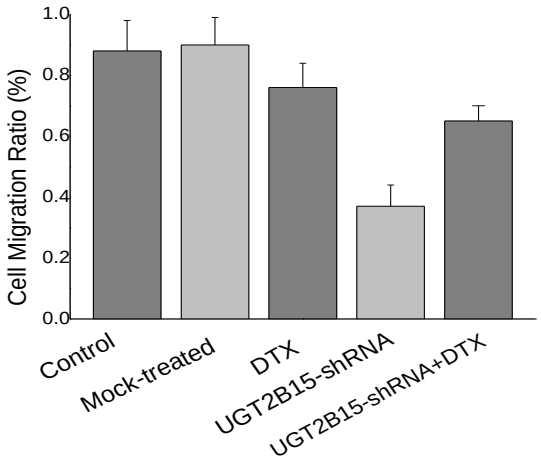

Figure 3. Efficacy of mock-treated shRNA, DTX, UGT2B15-targeted shRNA alone and the combination of UGT2B15-shRNA with DTX on DU145 cells migration. The summarized migration ratio (%) following 72 h treatment measured by wound healing assay
*Each column represents the mean ±SEM. **p<0.01*

DISCUSSION

Prostate cancer has become a major health problem in men worldwide, especially CRPC, for the poor prognosis and no efficient cure available. Chemotherapy is one of the most important treatments for CRPC, and the primary factor that has limited the success of chemotherapy in CRPC is the side effect and the emergence of drug resistance. Recent studies indicated that steroid-converting enzymes especially UGT2B15 plays a pivotal role in progression of CRPC [17]. Thus, we used UGT2B15-targeted shRNA to study the efficacy of UGT2B15 downregulation and in

combination with DTX on DU145 cells proliferation and migration. Our results showed that UGT2B15-targeted shRNA treatment caused a specific and profound decrease of UGT2B15 protein expression and the combinational treatment with DTX that was associated with decreased cell growth and cell migration whereas increased DTX sensitivity.

UGT2B15 has been shown to be involved in cisplatin resistance in colon cancers and is overexpressed in prostate cancer cells which may be involved CRPC [18]. The upregulation of UGT2B15 could contribute to intracellular synthesis of AR ligands and stimulation of cancer cell proliferation through AR signaling [14]. Tumor proliferation is associated with the low levels of intratumoral androgen driven primarily by elevated expression of enzymes responsible for androgen production. UGT2B15 plays a pivotal role in androgen biosynthesis within the prostate cancer which is thought to be involved in tumor progression, thus, combining UGT2B15-targeted shRNA with docetaxel may provide a potential effective therapy for human CRPC.

Studies have revealed that UGT2B15 involved in the conversion of adrenal androgens into testosterone, is often upregulated in CRPC which is consistent with our previous investigation (data not published). Inhibition of UGT2B15 enzyme activity holds promise for the treatment of CRPC patients [15]. However, one of the challenges in developing an UGT2B15 inhibitor is the gene similarity (>86%) with the other two subfamily members, AKR1C1 and AKR1C2 which will reduce DHT to less active metabolites, 3α-androstenadiol and 3β-androstanediol. Thus, inhibition of these AKR1C activities would inhibit DHT turnover and hence promote proliferative signaling in the prostate [19]. In the present study we used UGT2B15-targeted shRNA mediated UGT2B15 depletion in combination with DTX in DU145 cells and found that UGT2B15 inhibition induced a significant decrease in cell proliferation, suggesting that UGT2B15 is indeed involved in the regulation of cell viability in prostate cancer cells. Furthermore, these data also showed UGT2B15-targeted shRNA mediated downregulation of UGT2B15 significantly increased docetaxel activity against cell proliferation and migration in DU145 cells.

Our data demonstrate for the first time that shRNA-mediated UGT2B15 depletion augments the antitumoral effects of docetaxel, suggesting that UGT2B15 can be regarded as a promising target gene in genetic therapy for prostate cancer cells though the use of UGT2B15 shRNA for cancer therapy deserves additional investigation. The development of selective inhibitors targeting UGT2B15 and in combination with docetaxel treatment could represent a promising approach for effective antitumor therapy.

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