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Docetaxel enhances apoptosis and G2/M cell cycle arrest by suppressing mitogen-activated protein kinase signaling in human renal clear cell carcinoma

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ABSTRACT. Tremendous efforts have been made in renal cell carcinoma (RCC) patients' research; however, clinical findings in patients have been disappointing. The aims of our study were to identify better or alternative therapeutic methods that can reverse chemotherapy resistance and to enhance sensitivity to docetaxel (DOX)-based chemotherapy drugs. We evaluated the anti-proliferative effect of DOX against RCC cells. DOX was found to suppress proliferation of RCC cells under *in vitro* and *in vivo* settings. Flow cytometric analysis revealed that DOX suppressed cell growth by induction of both apoptosis and G2/M cell cycle arrest in a dose-dependent manner. Various patterns of gene expression were observed by cluster analysis. In addition, based on network analysis using the ingenuity pathway analysis software, DOX was found to suppress phosphorylation of extracellular signal-regulated kinase 1/2 and p38, suggesting that the mitogen-activated protein kinase signaling pathway plays a vital role in the anti-proliferative effect of DOX against RCC.

Key words: Docetaxel; Apoptosis; G2/M cell cycle; Human renal cell carcinoma

INTRODUCTION

Renal cell carcinoma (RCC) is a disease that can be effectively treated when cancerous cells are localized to the kidney. “Therapy” for RCC patients often includes surgical removal of the affected organ and the surrounding tissues with additional medical intervention (Gillett et al., 2005). However, if the disease has spread beyond the kidney capsule, the prognosis of the patients is generally poor. This poor outcome is in part due to the inherent ability of RCCs to resist various chemotherapies. Treatment strategies that are highly effective in reducing tumor masses and prolonging patient survival in other aggressive malignancies are ineffective in the case of RCC (Porta and Szczylik, 2009). Current therapies for VHL/clear cell carcinoma have focused on targeting the genes that are transcriptionally upregulated by HIF, such as vascular endothelial growth factor alpha, vascular endothelial growth factor receptor, platelet-derived growth factor receptor, as well as those involved in the mammalian target of rapamycin/hypoxia-inducible factor pathway. While most of these agents can induce responses in patients with advanced RCC, these responses are usually partial, and the disease continues to progress in most patients. The concept of using gene therapy to eliminate cancer cells has been explored for the last 20 years (Rein et al., 2006). The fundamental metabolic aspects of these cancer genes may be the Achilles’ heel that could potentially be used to develop more durable and effective forms of therapy.

The underlying biology of renal carcinomas has been extensively explored. With new advances in current technologies, the genetic and molecular factors contributing to this cancer can be further studied. Many critical discoveries have led to major innovations in RCC research, including a panel of molecularly targeted therapies. Unfortunately, despite often exciting pre-clinical data in many animal models, clinical findings in patients have been disappointing (Gabhann et al., 2010).

Docetaxel (DOX), a member of the taxane family of anti-cancer drugs, has been reported to exert anti-angiogenic effects (Wilson et al., 2008). DOX treatment increases Bcl-2 phosphorylation, downregulates Bcl-xL protein levels, and induces p53, thus leading to cell apoptosis (Caraglia et al., 2005; Parrondo et al., 2013). In symptomatic castration-resistant prostate cancer patients, DOX has been reported to be the first-line chemotherapeutic option (Atmaca et al., 2013) and has been shown to enhance the overall response and clinical remission of these patients (van Soest et al., 2013).

Cytotoxicity, especially peripheral neurotoxicity and hematopoietic side effects, are significant and inevitable side effects of DOX treatment (Maggioni et al., 2010; Vainas et al., 2012). In addition, during cancer metastases, drug resistance can develop through a variety of mechanisms, including inhibition of apoptosis and activation of extracellular signal-related PI3 kinase/Akt survival pathways (Jiang et al., 2009). Due to such resistance, DOX often fails to cure patients. It is therefore important to identify alternative therapeutic methods that can reverse chemotherapy resistance and enhance sensitivity to DOX-based chemotherapy drugs.

MATERIAL AND METHODS

Cell culture

Four human RCC cell lines (ACHN, A498, Caki-1, and NC 65) were cultured in RPMI-1640 medium (Gibco, Bio-cult, Glasgow, Scotland, UK) supplemented with 25 mM HEPES, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 mg/mL streptomycin,

and 10% heat-inactivated fetal bovine serum. Cell lines were maintained as monolayers in 10-cm plastic dishes, and incubated in 5% CO_2 at 37°C. Cells were treated for 3 days. DOX was purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA). Confluent cells were passaged with trypsin-EDTA (0.05% trypsin and 0.53 mM tetrasodium EDTA) and harvested for RNA isolation as described below.

Treatment and cell viability assay

RCC cells were seeded on 96-well plates in triplicates with Dulbecco's basal medium supplemented with 10% fetal bovine serum. Cells were maintained in culture for 24 h and treated with DOX (0.5, 1, 2, 4, and 8 nM). At the end of the incubation period, cell medium was removed, and 100 µL/well MTT solution (0.5 mg/mL in PBS) was added. The plates were incubated for 3 h at 37°C in the dark. After incubation at 37°C in 5% CO_2 for additional 4 h, the supernatants were carefully removed, and 150 µL DMSO was added to each well. The cells were then shocked for 10 min in the dark. Absorbance was measured at 450 nm via a Microplate Reader (Bio-Rad). Absorbance readings were analyzed to determine rates of cell proliferation and cytostasis, with untreated cells as controls.

RCC xenograft

Forty BALB/C nude mice, 3-4 weeks of age, were randomly divided into the following treatment groups, each containing 20 mice: control and DOX-treated mice. Four kinds of RCC cells (1×10^7 cells) were injected into the back region of each mouse. DOX (10 mg/kg) was injected into the peritoneal cavity three times a week when tumor diameter reached 5 mm. Control mice were injected with the same volume of water. Mice were observed for 5 weeks, and the diameter of each tumor was recorded. After 5 weeks, mice were sacrificed under deep anesthesia, and tumor volume was measured.

Flow cytometric analysis

RCC cell lines were cultured with DOX for 72 h. The cells were then collected, fixed, washed with phosphate-buffered saline, and incubated for 30 min in 7-AAD staining solution (BD Biosciences Pharmingen, San Diego, CA, USA). Cells in each phase of the cell cycle were analyzed, and cell counts were estimated using the FACSCalibur software (Becton Dickinson) and CellQuest (version 3.0).

RNA purification and cDNA preparation

Total RNA from cultured cells was extracted using Trizol reagent (Tiangen Biotech Co. Ltd., Peking, China) according to the manufacturer instructions. RNA concentrations were determined using a spectrophotometer. Genomic DNA from cultured cells was extracted using the DNeasy Blood & Tissue Kits (Qiagen), and DNA concentrations were measured with a NanoDrop spectrophotometer. Following denaturing gel electrophoresis, the samples were amplified and labeled using the Agilent Quick Amp labeling kit with Cy5 and Cy3 fluorescent dyes. All processes were carried out according to the manufacturer instructions.

cDNA microarray

Agilent SureHyb Hybridization chambers were used for hybridization with Agilent whole-genome oligo microarrays. After hybridization and washing, the processed slides were scanned with the Agilent DNA microarray scanner using settings recommended by Agilent Technologies. Analysis was performed after pre-treatment for scanning and normalization of the array data. Finally, the genes that may be relevant for DOX treatment were identified in our gene array screens.

Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed after the cells were cultured with DOX for 72 h. Antibodies against extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2, p38, and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA); anti-beta-actin monoclonal antibody (Abcam, Cambridge, UK) was used as an internal control. The immune complexes were detected using the electrogenerated chemiluminescence plus western blotting detection system (Amersham, Aylesbury, UK). Densitometry analysis was performed using the Scion Image software.

Statistical analysis

Data from all experiments were analyzed using the SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Results are reported as means ± SD. Two-sided Student *t*-test was used to analyze differences between groups. A P value < 0.05 was regarded as significant, whereas a P value <0.01 or <0.001 was considered highly significant.

RESULTS

Growth inhibition by DOX in RCC

The growth inhibitory effect of DOX on RCC cells was investigated, and we found that DOX suppressed proliferation of RCC cells in a dose-dependent manner *in vitro* (Figure 1A). As shown in Figure 1A, increased inhibition in RCC cell proliferation was observed on exposure to increased concentrations of DOX. Similar results were also confirmed *in vivo*; DOX suppressed the growth of RCC xenografts in BALB/C nude mice in a time-dependent manner. As demonstrated in Figure 1B, the average tumor volume in groups treated with DOX was lesser as compared to that in the control groups, after 5 weeks when mice were sacrificed and tumor weights were measured.

Apoptotic properties of RCC cells treated by DOX

In the initial study, the inhibitory effect of DOX on RCC cells was examined. Flow cytometric analysis revealed that DOX suppressed RCC cell growth by induction of apoptosis and G2/M cell cycle arrest in a dose-dependent manner (Figure 2A, B). Following DOX treatment, parts of the cells began to contract with increased inter-cell spacing, and cytoplasmic particle deposition was observed. In addition, as treatment time progressed, cell growth slowed, and an increased number of dead floating cells were observed in the media.

Table 1. Top 10 up- and downregulated genes under different conditions normalized to control.

Probe name	Gene symbol	GenBank accession No.	Map	Log2 ratio [DOX *vs* Cont]
TOP 10 genes upregulated by DOX				
A_23_P52067	GRHL3	NM_198173	chr1:24554121..24554180	4.71
A_23_P16523	GDF15	NM_004864	chr19:18360890..18360949	4.64
A_23_P1691	MMP1	NM_002421	chr11:102165966..102165907	4.37
A_23_P133408	CSF2	NM_000758	chr5:131439351..131439410	4.34
A_23_P144656	CDH10	NM_006727	chr5:24523256..24523197	4.33
A_23_P39766	GLS	NM_014905	chr2:191536067..191536126	4.23
A_23_P168882	TP53INP1	NM_033285	chr8:96007929..96007870	4.08
A_24_P262127	RRAD	NM_004165	chr16:65513735..65513676	3.97
A_24_P355816	DRAM	NM_018370	chr12:100841459..100841518	3.73
A_24_P326739	GLS2	NM_013267	chr12:55152246..55152187	3.63
TOP 10 genes downregulated by DOX				
A_24_P303815	UHRF2	NM_152896	chr9:6496080..6496139	-4.19
A_24_P124550	CCND1	NM_053056	chr11:69176451..69176510	-4.12
A_23_P138352	WNT2B	NM_004185	chr1:112864892..112864951	-3.50
A_23_P154217	ITGB6	NM_000888	chr2:160672479..160666576	-3.35
A_24_P363548	HIP1	NM_005338	chr7:75002247..75002188	-3.26
A_24_P206624	FGFR2	NM_022970	chr10:123233266..123233207	-3.17
A_24_P323434	CDCA2	NM_152562	chr8:25393501..25396834	-3.11
A_32_P77343	XRCC2	CR749256	chr7:151974055..151973996	-3.08
A_23_P382302	RIF1	NM_018151	chr2:152028268..152028327	-3.06
A_23_P356684	ANLN	NM_018685	chr7:36459112..36459171	-3.01

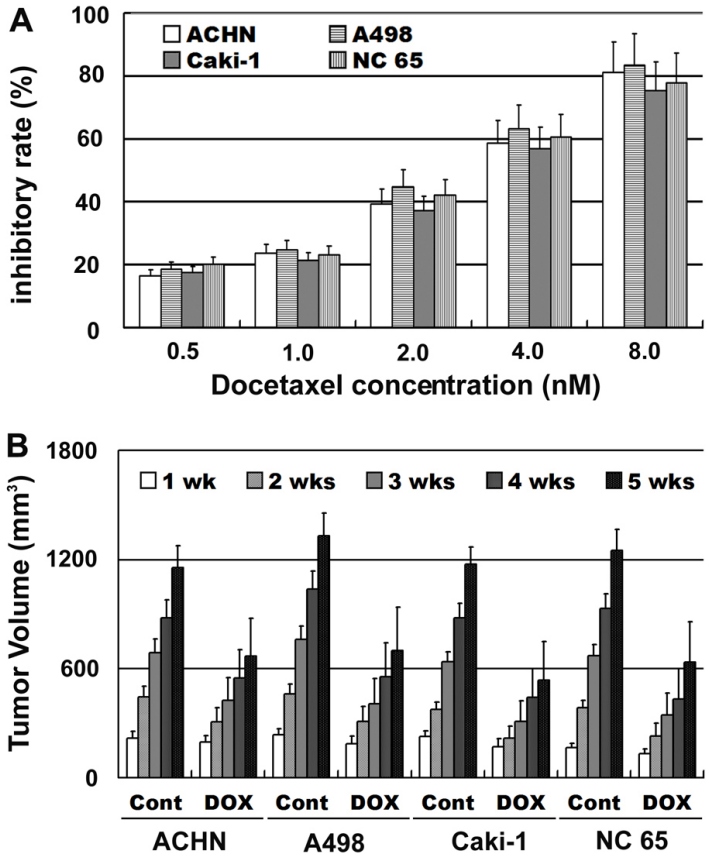

Figure 1. A. Growth inhibitory effect of docetaxel (DOX) on RCC cells *in vitro*. **B.** Average tumor volume in groups treated with DOX.

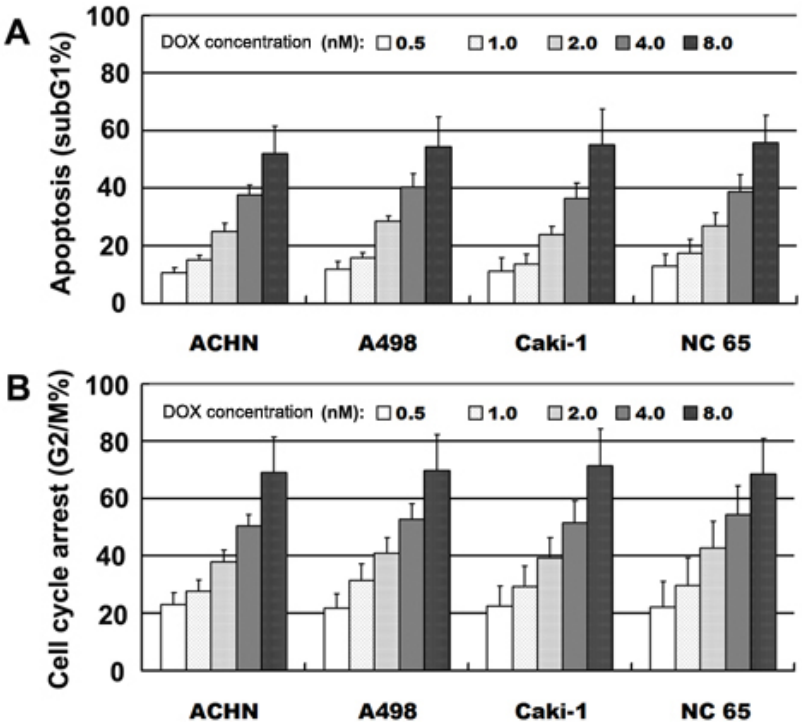

Figure 2. Dose-specific induction of apoptosis and G2/M cell cycle arrest by DOX.

cDNA microarray analysis

Hierarchical clustering of treatment with DOX showed distinguishable gene expression profiling among the samples. The top 10 up- and downregulated genes in the different conditions are shown in Table 1; the expression of each gene was calculated as the average of that for four RCC cell lines and has been presented as fold-changes normalized to the untreated control. We used ingenuity pathway analysis [(IPA) version 3.0] to search for possible biological pathways involved in DOX-induced RCC cell inhibition. The IPA software output was ranked in terms of probability, and dysregulated genes that were least likely to have occurred by chance would presumably be indicative of biologically relevant effects (Cariello et al., 2005). The canonical pathways involved in DOX were evaluated by their P values, and a low P value suggested that the pathway is highly correlated with activity of DOX against RCC cells (Table 2).

Suppression of the mitogen-activated protein kinase (MAPK) signaling pathway by DOX

To clarify how the MAPK signaling pathway is involved in the process of DOX-induced RCC cell inhibition, phosphorylation of the MAPK signaling pathway was evaluated after DOX treatment. In the two RCC cell lines (ACHN and A498), although DOX did not affect total expression of ERK1/2 or p38, it suppressed phosphorylation of ERK1/2 and p38 (Figure 3). These results suggested that the MAPK signaling pathway may play a vital part in the mechanism of action of DOX.

Table 2. Synergy-related pathways by regulated by DOX in RCC cells.

Pathways regulated by DOX	Number of entities	Matched with technology	Matched with entity list	P value
MAPK signaling	44	35	4	0.000
Cell cycle, mitotic	219	51	14	0.002
amb2 integrin signaling	56	20	6	0.002
Signaling by Aurora kinases	157	59	8	0.003
IL23-mediated signaling events	99	40	6	0.010
Cyclins and cell cycle regulation	26	15	4	0.011
p53 signaling pathway	14	11	3	0.011
Class I PI3K signaling events mediated by Akt	155	55	6	0.014
Signaling events mediated by HDAC class II	60	20	3	0.034
Integrin signaling pathway	28	21	3	0.039
Signaling mediated by p38-alpha and p38-beta	65	44	5	0.043
TNF alpha/NF-kB	214	191	14	0.043
Signaling events mediated by HDAC class I	186	73	6	0.045

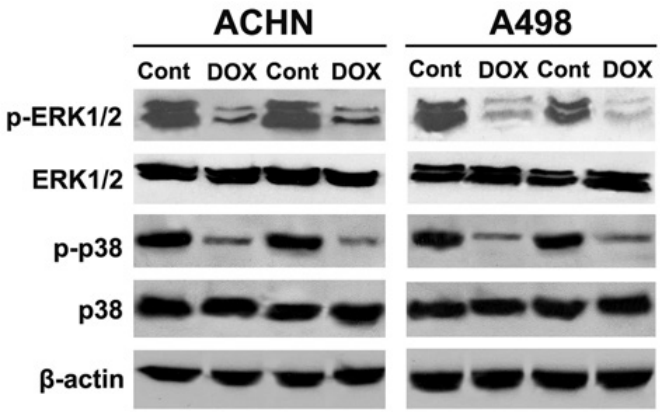

Figure 3. Suppression of ERK1/2 and p38 phosphorylation by DOX.

DISCUSSION

A large number of clinical trials involving chemotherapy regimens have been carried out in an attempt to overcome the limitations of current therapies for RCC. Among the current therapies, microtubule targeting-based chemotherapy is the most widely used method in treatment against RCC (Engels et al., 2005). However, long-term treatment often results in side effects and chemotherapy resistance, and usually contributes to variable clinical outcomes among patients with seemingly similar tumor types. Therefore, clinically effective chemotherapy regimens for RCC are required.

In an attempt to overcome drug resistance, we investigated the effective cytotoxic and apoptotic concentrations of the microtubule-targeting drug DOX in RCC cells. A number of genes potentially relevant to DOX treatment were identified in our gene array screens and merit detailed investigation. In the current study, we evaluated the anti-proliferative effect of DOX against RCC cells, and the results indicated that DOX suppressed proliferation of RCC cells under *in vitro* and *in vivo* settings. Flow cytometric analysis revealed that DOX suppressed cell growth by induction of both apoptosis and G2/M cell cycle arrest in a dose-dependent manner. Gene expression profile was analyzed using cDNA microarray analysis; several genes were up- or downregulated by DOX in RCC cells.

With respect to the diagnosis and prognosis of specific cancers, a variety of biomarkers are available (Henry and Hayes, 2012; Verma, 2012). There are, however, few biomarkers that are correlated with a general risk of cancer morbidity and mortality in healthy subjects. Grainyhead-like 3 (GRHL3) is a member of a highly conserved family of transcription factors critical for epidermal development and homeostasis across a wide range of species (Jane et al., 2005). Expression of this gene is largely confined to the surface ectoderm during embryogenesis. In adulthood, it is present in tissues that arise from the embryonic layer, including the skin and the lining of the oral cavity. Darido et al. (2011) also implicated GRHL3 in the pathogenesis of HNSCC in humans, providing possible alternate therapeutic strategies for targeting cancers that are often associated with extremely poor prognosis.

Growth-differentiation factor-15 (GDF-15) is a distant member of the transforming growth factor-beta cytokine superfamily. GDF-15 is expressed by many cell types in response to oxidative stress and inflammation, and seems to be involved in the regulation of apoptosis, cell proliferation, and cellular repair, which are biological processes that are key components of cardiovascular and cancer pathobiology (Breit et al., 2011; Xu et al., 2011). Human GDF-15 expression is controlled by p53, which is linked to atherosclerosis and cancer (Yang et al., 2003). It has been shown that GDF-15 level is elevated in several cancers, including prostate, ovarian, pancreatic, and colorectal cancers and multiple myeloma (Staff et al., 2010; Breit et al., 2011; Wallin et al., 2011; Brown et al., 2003, 2009, 2012; Corre et al., 2012). In some cancer types, elevated levels of GDF-15 have been associated with adverse prognosis (Brown et al., 2009; Staff et al., 2010). Therefore, GDF-15 may merit further investigations, and may have the potential to be used for preventive measures against cancer.

UHRF2 was originally referred to as NIRF and was cloned through a two-hybrid interaction with PEST-containing nuclear protein. It was described to be highly expressed in proliferating cells, but not during the G0/G1 cell cycle phase (Mori et al., 2002). Subsequent study demonstrated that ectopic UHRF2 expression causes G1 phase arrest concomitant with Cdk2-cyclin E binding and degradation (Li et al., 2004). UHRF2 has also been reported to interact with many cell cycle regulators, including CDKs, cyclins, proliferating cell nuclear antigen, p53, and pRB (Mori et al., 2011). Some of these interactions, such as those with cyclin D1 and E1, destabilize the target proteins. The mechanisms by which UHRF2 suppresses various tumor types, whether or not it involves the suppression of Rb/E2F1-associated apoptosis and/or G1 arrest, and how UHRF2 controls E2F1 induction of the target gene are critical questions that need to be further explored.

We also used IPA to search for possible biological pathways during RCC cell inhibition by DOX. IPA analysis showed that several implicated genes have the potential to code for proteins that are involved in the MAPK signaling pathway. Signal transduction via MAPKs plays a key role in a variety of cellular responses, including cell proliferation, differentiation, and death (Wagner and Nebreda, 2009). In accordance with our results, inhibition of the p38 MAPK pathway has been reported to decrease survival and increase apoptosis in several cancer cell lines (Kumar et al., 2009). Our results indicated that although DOX did not affect total expression of ERK1/2 or p38, it suppressed ERK1/2 and p38 phosphorylation, suggesting that the MAPK signaling pathway plays a vital role in the anti-proliferative effect of DOX against RCC.

In conclusion, our study suggested that treatment of DOX in RCC cells leads to growth inhibition, and that DOX may enhance apoptosis and G2/M cell cycle arrest by suppressing the MAPK signaling pathway. Although our results need to be further examined in future studies, they suggest that targeting microtubules by DOX may be a useful approach to improve outcomes in RCC.

Conflicts of interest

The authors declare no conflict of interest.

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