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## TBX3 Role in Colorectal Cancer Treatment

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### Abstract

The T-box family of developmentally important transcription factors has been shown to play a role in the genesis of cancer and shows much promise for a targeted therapeutic approach. For example, the T-box factor TBX3 is overexpressed in a number of cancers including colorectal cancer but its precise role in the treatment of this disease still need to be clarified. We provide novel evidence to show that knocking down TBX3 sensitizes colorectal cancer cells to cisplatin treatment.

### Keywords:

Cancer,  
Cisplatin,  
TBX3.

### 1. Introduction:

Cancer is a leading cause of death in both developing and less developed countries (Torre et al., 2015). Globally, colorectal cancer (CRC) is the third mostly diagnosed type of cancer amongst males, and the second in females (Torre et al., 2015). In Gaza however, colon cancer is the second mostly diagnosed type of cancer representing 10% of cancer cases, the first mostly diagnosed in males, the second mostly diagnosed in females (Bitar, 2015).

The high incidence rate of CRC is attributed to a number of factors, including unhealthy lifestyle, growth, and aging of the population (Torre et al., 2015). The impact of the disease extends to affect the economic, social, psychological aspects of the quality of life in cancer patients.

A crucial step to reduce CRC mortality is the detection of early-stage cancer and precancerous lesions (Huang et al., 2010). Several tests or procedures are used for the early detection of CRC including fecal occult blood test, flexible sigmoidoscopy, double contrast barium enema, and colonoscopy (Smith, Cokkinides, & Eyre, 2006). However, because of their low adherence rates, high

cost or low sensitivity, none of these methods is regarded as a well-accepted screening tool (Huang et al., 2010). Despite the improvements in these screening methods, only 30% to 40% of CRC patients are diagnosed at an early stage. For a screening method to be considered ideal, it should have a high sensitivity and specificity for early-stage CRC and precancerous lesions. In addition, this method should be safe and affordable so that it can be broadly accepted by patients and oncologists (Huang et al., 2010). Thus, an effective and safe early stage screening methods would have substantial clinical benefits, and would reduce the mortality of patients with CRC (Bo et al., 2009).

The identification of transcription factors which play a key role in colorectal cancer progression is important because they may represent good diagnostic biomarkers and targets in the development of novel therapeutic approaches to treat this cancer. The rationale for this is based on the principle that transcription factors are ultimately responsible for controlling gene expression patterns resulting in tumor formation,

progression and metastasis. In the last decade, several transcription factors with key roles in colorectal cancer progression were identified as potential therapeutic targets.

The T-box family of transcription factors are important developmental regulators and have been shown to contribute to several human syndromes (Naiche, Harrelson, Kelly, & Papaioannou, 2005; Peres & Prince, 2013). In addition to their key role in development, extensive investigations suggest that over expression of some T-box factors, including TBX2 and TBX3 may drive cancer (American Cancer Society, 2013; Bilican & Goding, 2006; Burgucu et al., 2012; Demay et al., 2007; Hoogaars et al., 2008; Humtsoe et al., 2012; Liu, Jiang, & Zhang, 2010; Peres et al., 2010; Peres & Prince, 2013). Both transcription factors are up-regulated in a number of cancers including melanoma (Hoek et al., 2004) breast cancer (Wu et al., 2001) and Colorectal cancer (Shan et al., 2015) where they were shown to be required for tumour formation and cell migration (Jacobs et al., 2000; Peres & Prince, 2013; Redmond et al., 2010; Yu et al., 2010). Importantly, knocking down TBX2 and TBX3 was shown to reverse key features of the melanoma and breast cancer phenotype suggesting that it may be a useful target in the development of novel anti-cancer drugs to treat these cancers (Peres et al., 2010; Wansleben, Davis, Peres, & Prince, 2013). Furthermore, silencing Tbx3 in rat bladder carcinoma cells rendered the cells sensitive to doxorubicin-induced apoptosis and the overexpression of TBX3 was associated with a chemotherapy-resistant phenotype (Claire-Angélique Renard et al., 2007). Similar findings showed that knocking down TBX3 sensitized human colorectal carcinoma cells to doxorubicin via activating the p14- p53 pathway (Zhang et al., 2011). Taken together these findings suggest that TBX2 and/or TBX3 are promising molecular markers and their depleting as new targets in cancers where they are over expressed might enhance the anti-cancer activity of chemotherapeutic drugs.

The current study therefore tested the possible role of TBX3 in response to cisplatin chemotherapeutic treatment in colorectal cancer cells. Our results demonstrate that cisplatin treatment initially increases TBX3 level but Moreover, the current results show that knocking down tbx3 enhances cisplatin cytotoxic effect which might suggests anti-chemotherapeutic role of TBX3 in colorectal cancer.

## 2. Material and methods:

### 2.1 Cell culture:

The human cell line HT-29 colorectal cancer cells were maintained in RPMI 1640 medium (Highveld Biological, Lynd-hurst, UK). Media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37°C in a 5% CO<sub>2</sub> and 95% air-humidified incubator. Media was replaced every 2-3 days and cells were routinely subjected to mycoplasma tests. Only mycoplasma free cells were used in experiments.

### 2.2 Cisplatin treatment:

Cisplatin (CDDP) (Pfizer, South Africa) was used as a positive control for melanoma treatment (Matin et al., 2013; Mirmohammadsadegh et al., 2007). Cells were treated with CDDP at specific concentrations as indicated in each experiment. In all cases untreated cells were incubated with 150 mM NaCl (the vehicle in which CDDP was dissolved in). Cells were incubated in the dark at 37°C.

### 2.3 Cytotoxicity assays:

To determine the cytotoxic effect of the indicated compounds and drugs, cells were seeded (3000-6000 cells/well) in quadruplicate in a 96-well plate and treated after two days with a range of the indicated concentrations of specific compounds or vehicles for 48 hours. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay according to the manufacturer's instructions (Roche, USA). Briefly, 10 µL of MTT solution was added to each well and incubated for 4 hours at 37°C. This was followed by the addition of 100 µL solubilisation buffer (10% SDS in 0.01 M HCl) and incubated overnight at 37°C. Absorbance (585 nm) was then determined for each well and the mean cell viability was calculated as a percentage of the control. Three separate experiments were performed to determine the concentration of cisplatin required to kill 50% of the cells (IC<sub>50</sub>). The IC<sub>50</sub> values were calculated from sigmoidal plots with GraphPad Prism version 5.

### 2.4 Western blot analysis:

Cells were washed twice with ice-cold PBS and collected by scraping with a 1 ml plunger. Whole cell extracts were prepared using 2X Laemmli sample buffer (see appendix, section 6.6), boiled for 10 min and stored at -20°C. Alternatively, whole cell extracts prepared from cells using RIPA buffer were stored on ice for 30 min and collected by centrifugation at 12 000

rpm for 20 min at 4°C. The protein concentration for each cell extract was determined using the BCA Protein Assay kit (Pierce, USA), with bovine serum albumin as the standard. Equal amounts of protein were loaded in each lane and resolved on 6-15 % SDS-PAGE gels and then transferred electrophoretically to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, USA). Membranes were blocked for 1 hour at room temperature with PBS containing 5% non-fat dry milk and probed with appropriate primary antibodies O/N at 4°C with shaking. Membranes were washed in PBS containing 0.1% Tween 20 (PBS/T) and incubated with either donkey anti-goat (Santa Cruz Biotechnology, CA, USA), goat anti-mouse or goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (1:5000) (BioRad, Hercules, CA, USA) in blocking solution at room temperature with shaking for 1 hour. Membranes were again washed in PBS/T and visualized by enhanced chemiluminescence (Pierce, USA).

### 2.5 Quantitative real-time PCR (qRT-PCR):

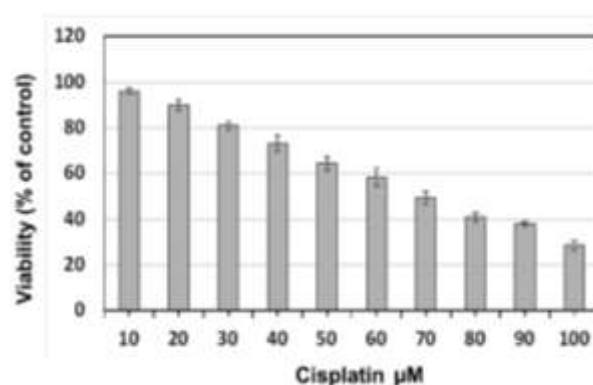
Total RNA was extracted from cells using the RNeasy Plus Mini kit (Qiagen, USA). The quality and concentration of RNA was determined by spectrophotometry. Only samples exhibiting an A260/A280 ratio equal to or greater than 1.8 were selected and stored at -80°C for further applications. Reverse transcription of RNA was performed according to the manufacturer's instructions using the InProm-IITM reverse transcription system (Promega, USA). Briefly, 1 µg of RNA was combined with 0.5 µg of Oligo (dT)15 primer in a 5 µl volume and denatured at 70°C for 5 min, chilled on ice and combined with reverse transcription reaction mix (1X ImProm-IITM Reaction buffer, 3 mM MgCl<sub>2</sub>, 0.5 mM dNTP mix, 20 units RNasin® ribonuclease inhibitor and 1 µl of ImProm-II TM reverse transcriptase) to a final volume of 20 µl. After a brief annealing at 25°C for 5 min, the reactions were incubated at 42°C for 1 hour, followed by 15 min incubation at 70°C to inactivate the reverse transcriptase prior to PCR. qRT-PCR was conducted in 96 well sealed plates on an Applied Biosystems StepOne Plus thermal cycler using 2x SYBR green master mix (Applied Biosystems, Carlsbad, CA, USA), a final concentration of 0.3 µM of each primer and 2 µl of cDNA in a total volume of 10 µl. PCR cycle parameters were: denaturation for 15 min at 95°C, combined annealing and extension for 35 cycles at 60°C for 1 min. Each DNA sample was quantified in triplicate and a negative control without cDNA template was run with

every assay to assess the overall specificity. Melting curve analyses was carried out to ensure product specificity. Relative mRNA expression levels were normalised to human glucuronidase beta (GUSB) or human beta-actin using the 2-ΔΔCt method. The Microsoft Excel program was used to calculate the standard deviation and statistically significant differences between samples using the Student *t* test. *P* values of <0.05 were considered statistically relevant.

## 3. Results:

### 3.1 Cisplatin exerts a moderate cytotoxic effect against HT-29:

To investigate the cytotoxic effect of cisplatin on colorectal cancer cells, the HT-29 cells were treated with a range of cisplatin (0 to 100 µM) for 48 hours and the MTT assay was used to determine cell viability. The concentration that inhibits cell growth by 50 % (IC<sub>50</sub>) was calculated to be 60.8 µM of cisplatin (Figure 1). Importantly, previous studies on other cancer cells showed that cisplatin exerts its cytotoxic effect at much lower concentrations for example its IC<sub>50</sub> on breast cancer cells (MCF7) and melanoma cells (ME1402) was about 10 µM (Aliwaini et al., 2015; Aliwaini, Swarts, Blanckenberg, Mapolie, & Prince, 2013). These results indicate that the colorectal cell line HT-29 is slightly resistant to cisplatin treatment.



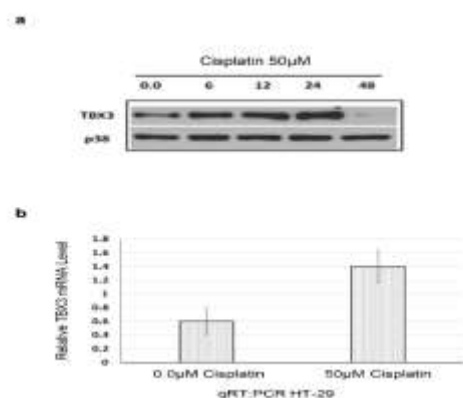
**Figure 1** Cisplatin induces a moderate cytotoxic effect on colorectal cancer cells

HT-29 cells were plated in 96-well plates and after 48 hours the cells were treated with increasing concentrations of cisplatin (0-100 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment. The graphs represent the mean percentage ± SEM of surviving treated cells

relative to control cells of at least three independent experiments performed in quadruplicate.

### 3.2 Cisplatin up-regulates TBX3 in HT-29:

The transcription factor TBX3 is up-regulated in a group of cancers including breast and colorectal cancers (Hoek et al., 2004; Shan et al., 2015; Wu et al., 2001) and knocking down TBX3 was shown to be a useful strategy to enhance chemotherapeutic cytotoxicity. Based on the above reports we hypothesized that in response to chemotherapeutic agents TBX3 levels may be up-regulated resulting in cancer cells being less sensitive to these anti-cancer treatments. To explore this possibility the effect of cisplatin on TBX3 levels was firstly determined. Briefly, HT-29 cells were treated with 0.50  $\mu\text{M}$  cisplatin 0.0, 6, 12, 24 and 48 hours and total protein harvested and subjected to western blotting with an antibody to TBX3. The results obtained revealed that in response to cisplatin treatment TBX3 levels increased in a time-dependent manner in HT-29 cells (Figure 2-a). To investigate whether cisplatin up-regulates TBX3 transcriptionally or post-transcriptionally, HT-29 cells were treated with 50  $\mu\text{M}$  cisplatin for 6 hours and TBX3 expression was measured by quantitative real time PCR (qRT-PCR) analysis. The results show that cisplatin increased mRNA TBX3 level which suggests that cisplatin may up-regulate TBX3 transcriptionally (Figure 2-b).



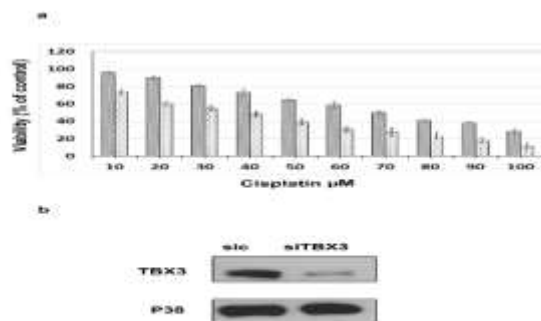
**Figure 2** Cisplatin treatment up-regulates TBX3 protein levels

**(a)** Cisplatin treatment increases TBX3 levels in HT-29 cells. Protein extract from Ht-29 cells treated with vehicle or 50  $\mu\text{M}$  cisplatin for 24 and 48 hours were analyzed by SDS-PAGE (8%) and western blotting using an antibody to TBX3. p38 was used as a loading control.

**(b)** Cisplatin up-regulates TBX3 at a transcriptional level, cells were pre-treated with vehicle or 50  $\mu\text{M}$  cisplatin and relative TBX3 mRNA levels were quantitated using qRT-PCR and normalized to human GUSB. Results represent the average of biological replicates. Error bars =  $\pm$ SEM.

### 3.3 Knocking down TBX3 enhanced cisplatin induced cytotoxicity:

To investigate the potential role of TBX3 depletion in cisplatin-induced cytotoxicity, HT-29 cells were transiently transfected with non-silencing siRNA (sictrl) or TBX3-specific siRNA (si-TBX3). Transfected cells were treated with a range of cisplatin doses (0 to 100  $\mu\text{M}$ ) for 48 hours and the MTT assay was used to determine cell viability. Figure 3-b confirms that the HT-29 siTBX3 cells used in this experiment did indeed have lower levels of TBX3 than the sictrl cells and Figure 3-A shows that the siTBX3 cells were more sensitive to cisplatin than the sictrl cells. While 50  $\mu\text{M}$  of cisplatin killed about 40% of sictrl cells, the same concentrations killed 60% of siTBX3 cells. These results indicate that knocking down TBX3 might enhance the cytotoxic effect of cisplatin in HT-29 cells.



**Figure 3** Knocking down TBX3 in HT-29 cells enhances cisplatin cytotoxic effect

**(a)** HT-29 siTBX3 cells are more sensitive to cisplatin than HT-29 sictrl cells. Cells were plated in 96-well plates and cell viability was assessed by the methylthiazol tetrazolium (MTT) assay over 24 hours of cisplatin treatment. The graph represents the mean percentage  $\pm$  SEM of untreated cells of at least three independent experiments performed in quadruplicate. A Microsoft Excel student's *t* test was performed to calculate statistical significance ( $*p < 0.05$ ). **(b)** Western blot of protein from HT-29 sictrl and HT-29 shiBX3

cells shows successful TBX3 knockdown in HT-29 sitBX3 cells.

#### 4. Discussion:

The highly homologous TBX3 protein, a member of T-box family, has been implicated in the progression of a number of cancers including a subset of colorectal cancers (Hoek et al., 2004; Hoogaars et al., 2008; Peres et al., 2010; Shan et al., 2015; Vance, Carreira, Brosch, & Goding, 2005). Tbx3 overexpression has been shown to be sufficient to immortalize mouse embryonic fibroblasts in a process involving the repression of the cell cycle regulator p19<sup>ARF</sup> (Brummelkamp, Kortlever, et al., 2002; Brummelkamp, Bernards, & Agami, 2002). Two studies later showed that TBX3 interacts with histone deacetylases to repress p14<sup>ARF</sup>, the human homolog of p19<sup>ARF</sup>, in human breast cancer cells where it is overexpressed (Fan, Huang, Chen, Gray, & Huang, 2004; Yarosh et al., 2008). The current study shows that knocking down TBX3 led to the sensitisation of the HT-29 colorectal cancer cells to cisplatin. In particular, depleting TBX3 in Ht-29 cells resulted in an increase in cell death. This is in line with the results from a previous study which showed that TBX3 is overexpressed in a subset of drug resistant hepatoblastomas and that its inhibition enhanced doxorubicin-induced apoptosis (C-A Renard et al., 2007). Furthermore, Tbx3 was shown to suppress apoptosis by preventing the induction of p53 and its target p19<sup>ARF</sup> in mouse embryo fibroblasts overexpressing Myc and Ras (Carlson, Ota, Song, Chen, & Hurlin, 2002). In light of these observations, the current study showed that TBX3 may be inhibiting the cell death in response to cisplatin and might be a cause of drug resistance. Importantly, the data provide compelling evidence in support of targeting TBX3 in combination with cisplatin as a viable option for treatment of TBX3-driven colorectal cancer.

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### دور عامل النسخ TBX3 في علاج سرطان القولون

**كلمات مفتاحية:**  
 TBX3 عامل نسخ،  
 سرطان القولون،  
 سيسبلاتين.

تعتبر عوامل النسخ TBOX مهمة لنمو الأجنة وتطورها وكذلك أشارت الدراسات السابقة إلى دور هذه العوامل في الأمراض وخاصة مرض السرطان. وعلى سبيل الخصوص فإن عامل النسخ TBX3 يوجد بشكل مرتفع في عدة أنواع من السرطانات منها سرطان الثدي والقولون والبروستاتا. وعلى الرغم من ذلك فلا تزال العديد من التساؤلات مطروحة حول دور هذا العامل في علاج السرطانات. الدراسة الحالية تضع مؤشرات أولية حول إمكانية لعب هذا العامل TBX3 دوراً ما في علاج سرطان القولون. على الرغم من عدم الوضوح في هذا الدور إلا أن الأرجح أن هذا البروتين يؤثر بزيادة مقاومة السرطان للعلاجات.