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Cisplatin Induces Down Regulation of BCL2 in T47D Breast Cancer Cell Line

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Abstract: Over expression of the anti-apoptotic protein BCL2 has been associated with several malignancies. BCL2 is a prognostic marker in breast cancer. The BCL2 protein is a highly conserved member of the BCL2 family and constitutes an important regulator of apoptosis. Apoptosis is the main response of cells to chemotherapeutic agents including cisplatin. In this study, T47D cells were treated with various concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25, 50, 100μM) for 24h. Then, cell viability was assessed using MTT assay and IC50 was determined. RNA was extracted and cDNA was synthesized. In the present study, *BCL2* was chosen as target and *TBP* was chosen as internal control gene, respectively. Specific primers were designed by primer express software v.3.0. target /control gene expression ratios were calculated by $2^{-\Delta\Delta Ct}$ formula. The Real-time PCR results showed that *BCL2* gene expressions were decreased. The results showed that cisplatin exerted a dose-dependent inhibitory effect on the viability, via down regulation of *BCL2* gene, in T47D cells.

Keywords: BCL2; Cisplatin; Apoptosis; Breast Cancer

Introduction

Cancer killed 7.9 million people worldwide in 2007 (1). Breast cancer is one of the leading causes of women mortality worldwide. Chemotherapy is the only option for treating the malignant breast cancer and condition for increases the life span of the patient (2). Chemotherapeutic agents have been developed to counter the continuing breast cancer problem. However, most chemotherapeutic drugs effectively target rapidly dividing cells causing damage and are thus referred to as "cytotoxic drugs." Cisplatin is an important chemotherapeutic agent used widely for the treatment of a variety of malignancies, including breast, testicular, ovarian, cervical, prostate, head and neck, bladder, lung and refractory non-Hodgkin's lymphomas (3, 4). The cytotoxic effect is likely a result of inhibition of replication by cisplatin-DNA adducts and induction of apoptosis. However, the events leading to apoptotic cell death after cisplatin treatment are not well understood and may involve regulation of the expression of multiple genes (5). BCL2 is a prognostic marker in breast cancer. The BCL2 protein is a highly conserved member of the BCL2 family and constitutes an important regulator of apoptosis (6). In this study, we evaluated the expression of *BCL2* gene T47D cell line incubated with different concentrations of cisplatin at 24h.

Materials and methods

Cell culture and cisplatin treatment

T47D cell line was obtained from National Cell Bank of Iran. T47D cell was cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin – streptomycin under standard culture conditions (37°C, 95% humidified air and 5% CO_2). The cells were incubated with different concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25, 50 and 100μM) at 24h. Each concentration of cisplatin was tested on 3 wells of the 96-well plates containing 1×10^4 T47D cell (7).

Total RNA extraction and cDNA synthesis

RNA was extracted by RNX solution. cDNA synthesis was performed using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, USA) .

Agarose gel electrophoresis

The PCR products were resolved by electrophoresis in 1.5% Agarose gel in 0.5X TBE (Tris-Borate-EDTA) buffer. 100 bp ladder was used as molecular weight marker. After staining the gel with ethidium bromide, fragments were visualized

by UV transilluminator and photographed. Gel electophoresis was carried out to confirm the primers specificity and amplification of PCR products.

Real-time RT-PCR

Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) was employed to perform quantitative PCR on the Rotor-Gene 6000 (Corbett Research, Australia) applying the following thermal-cycling conditions: 10 min at 95˚C (1 repeat) as first denaturation and Hot-start enzyme activation, followed by 40 cycles at 95˚C for 15s and 60˚C for 1 min. Each complete amplification stage was followed by a melting stage; at 95˚C for 15s, 60˚C for 30s and 95˚C for 15s. The PCR amplification was performed in 25µl reaction containing 12.5µl Power SYBR Green PCR Master Mix (2x), 1µl Forward primer (0.4µM), 1µl Reverse primer (0.4µM), 5µl first-strand cDNA (100ng) and 5.5µl double-distillated water. Standard curve was obtained by plotting Ct values against log cDNA concentrations of five serial two-fold dilutions of the target nucleic acid. The serial dilutions were 75, 150, 300, 600 and 1200ng/µl of standard cDNA used. It was used to find out the dynamic range of the target and reference genes, to calculate the slope (PCR efficiency), r and R^2 coefficients, precision (standard deviation) and sensitivity (y-intercept). The efficiency of the reaction was calculated by the following equation: $E = [10^{(-1/slope)} - 1]$.

Quantitative data analysis of Real-time PCR

Calculation of the gene expression was carried out using comparative threshold cycle (Ct). The mean threshold cycle (mCt) was obtained from triplicate amplifications during the exponential phase. Then, mCt value of reference gene (*TBP*) was subtracted from mCt value of the target gene (*BCL2* gene) to obtain ΔCt and ΔΔCt values of each sample were calculated from corresponding Ct values; where $\Delta\Delta Ct = [\text{mCt target - mCt reference}]_{\text{(treated sample)}} - [\text{mCt target - mCt reference}]_{\text{(untreated sample)}}$. Finally, *BCL2* gene expression/*TBP* gene expression ratio was calculated using the ratio formula (ratio = $2^{-\Delta\Delta Ct}$).

Statistical analysis

Data are expressed as mean, standard deviation, correlation coefficients (R^2) and graph preparation were done using Microsoft Office Excel 2007 software to estimate the reproducibility of the assay. The *P*-value of <0.05 was considered statistically significant for student's *t*-test analysis.

Results

Cisplatin cytotoxicity on T47D cell

Different concentrations of cisplatin (3.125, 6.25, 12.5, 25, 50 and 100µM) at 24h have cytotoxicity effect on breast cancer cell. Compared to the controls, the lower dose of cisplatin (3.125µM) 6.14% (difference not significant, $p>0.05$) and the higher dose of cisplatin (100µM) 59.59% ($p<0.001$) decreased in total cell number (Fig. 1). IC50 of cisplatin after 24h was calculated to be 43.694µM.

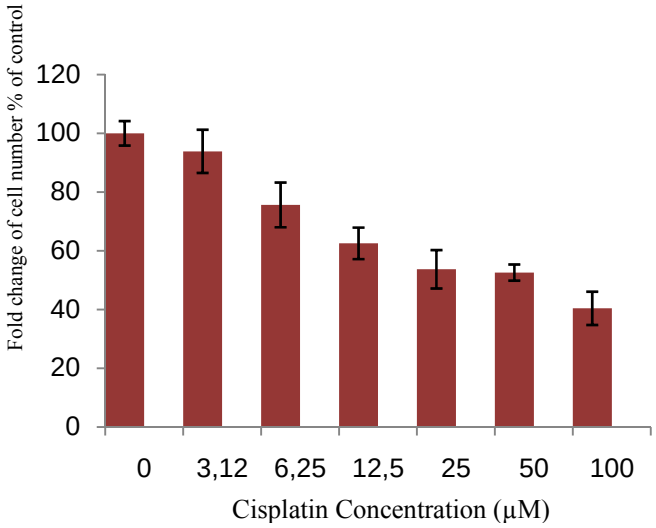

Figure1: Cytotoxiciy of cisplatin on MCF-7 cells. Results are expressed as a percentage of viability compared to control and are presented as mean±SD from at least three independent experiments

Absolute quantification analysis and PCR efficiency

We used different concentrations of cDNA for *BCL2* and *TBP* genes to prepare standard curves. Dynamic range of *BCL2* and *TBP* genes were obtained from 75 to 1200 ng/µl. The slope of standard curves were -3.42 (*BCL2*) and -3.36 (*TBP*). Then, PCR efficiency was calculated 96.01% for *BCL2* and 98.4% for *TBP* (Fig. 2).

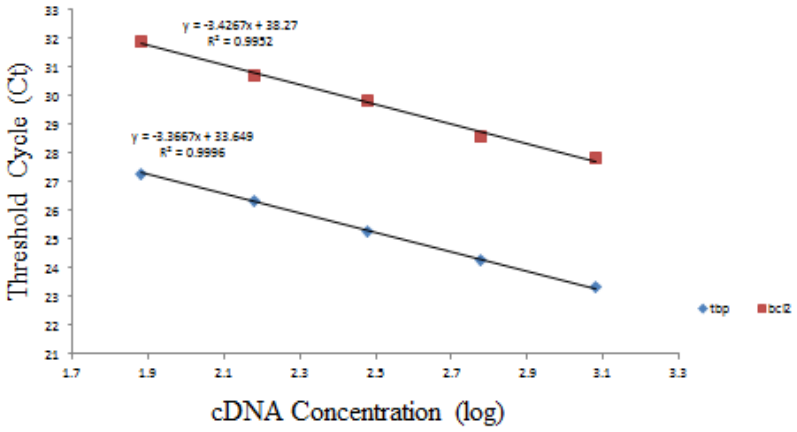

Figure 2. Standard curve is generated by plotting Ct values against the logarithm of the cDNA concentration. Standard curve of TBP: slope = -3.36, y-intercept = 33.649, R^2= 0.999. Standard curve of BCL2: slope = -3.42, y-intercept = 38.27, R^2= 0.998.

Melting curve analysis and gel electrophoresis

The melting curve was drawn based on the temperature (x axis) and dF/dT derivation (y axis). The reproducibility of a melting curve is high with a standard deviation of only 0.1°C between runs. It was generated to screen for primer dimmers and to document single product formation for each gene. The melting peaks have been drawn at 79.5°C for *TBP* gene and 83.8°C for *BCL2* gene (Fig. 3A). Gel electrophoresis results showed specific amplification sequence of interest (Fig. 3B).

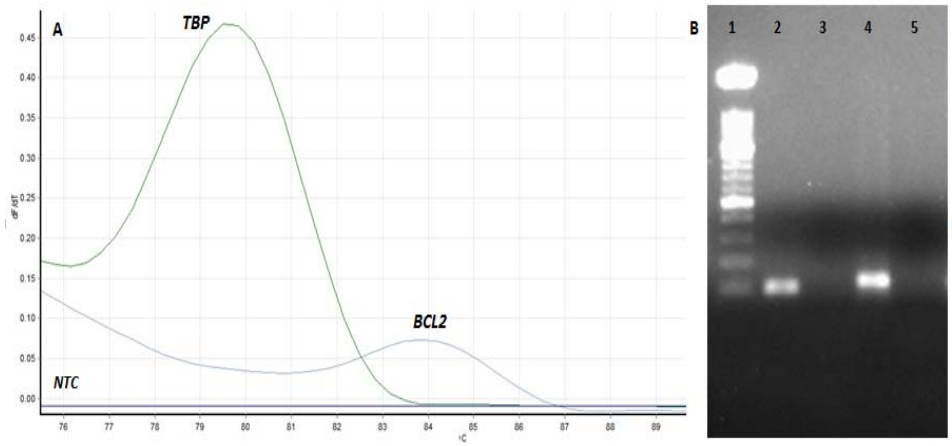

Figure3 (A) The melting peaks at 79.5°C for *TBP* gene (1) and 83.8°C for *BCL2* gene (2) indicate the specific products that melt at the different temperature. Flat peak demonstrates Non Template Control (3). (B) Lane 1 : Molecular Size marker - 100 bp ladder. Lane 2 : 96 bp PCR product of *TBP* gene. Lane 3 : NTC for *TBP* gene. Lane 4 : 122 bp PCR product of *BCL2* gene. Lane 5 : NTC for *BCL2* gene.

Relative quantification analysis using amplification plots

The relative gene expression between two samples (treated and untreated) can be determined by the difference in their Ct values of exponential phase. The mCt value for *TBP* gene was 22.27 in different concentrations of cisplatin. The *BCL2*/*TBP* gene expression ratio equals to $2^{-\Delta\Delta Ct}$. The $2^{-\Delta\Delta Ct}$ were calculated as 0.07± 0.01 ($p< 0.01$) in 43.694μM of cisplatin.

Discussion

Each year, over a million women worldwide are diagnosed with breast cancer, accounting for 25% of all female cancers. Treatments include surgery, radiation therapy, chemotherapy, etc (8). Cisplatin is one of the most widely used antineoplastic alkylating agents for the treatment of certain cancers. It was discovered fortuitously by Dr. Rosenberg in 1965 (9). DNA is thought to be the primary biological target of cisplatin. The control of apoptosis involves a large variety of proteins. BCL2 family proteins regulate DNA damage-induced apoptosis by regulating the release of mitochondrial cytochrome c in response to DNA damage. Different members of the BCL2 family either protect cells from cell death (for example, BCL2, BCL-XL, and BCL-W) or promote cell death (for example, BCL-XS, BAD, BAK, BAX and BIK), and undergo homo- and hetero dimerization, depending upon their expression levels (10). BCL2 family proteins regulate DNA damage-induced apoptosis by regulating the release of mitochondrial cytochrome c in response to DNA damage. It is possible that the *BCL2* gene plays a role in breast cancer development since it is overexpressed in 70% of breast cancer specimens (11). The product of the *BCL2* gene is known to play a role in promoting cell survival and inhibiting apoptosis in a variety of cases, while the related protein *BAX* accelerates apoptosis and counters the apoptosis repressor function of *BCL2*.
Many researchers showed that cisplatin downregulated BCL2 mRNA levels in MCF-7 cell line (12). Other studies showed that BCL2, BCL-XL, BAD and BAX were differentially regulated by cisplatin (6). Our results showed that cisplatin exerted a dose-dependent inhibitory effect on the viability, via down regulation of *BCL2* gene, in T47D cells.

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