

Research Article

Cisplatin Induces Up-Regulation of KAI1, a Metastasis Suppressor Gene, in MCF-7 Breast Cancer Cell Line

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Abstract

Purpose: To investigate the effect of cisplatin on cell toxicity and metastasis through modulation of KAI1 gene expression.

Methods: MCF-7 cells were incubated with different concentrations of cisplatin for 24 h. RNA was extracted by trizol and cDNA synthesized. KAI1 and TBP were chosen as target and internal control genes, respectively. Specific primers were designed by primer express software, v.3.0. KAI1/TBP and gene expression ratio was calculated using the formula, $2^{-\Delta\Delta Ct}$.

Results: Cisplatin exerted a dose-dependent inhibitory effect on the viability of highly metastatic MCF-7 cells. KAI1/TBP gene expression ratios were 1.97 ± 0.19 ($p < 0.05$), 2.96 ± 0.55 ($p < 0.05$), 9.06 ± 0.27 ($p < 0.001$) and 12.38 ± 0.88 ($p < 0.01$) in 10, 20, 50 and 100 μ M concentrations of cisplatin.

Conclusion: These findings indicate that cisplatin can inhibit metastasis by up-regulating KAI1 gene in MCF-7 cells.

Keywords: Cisplatin; KAI1; Metastasis; Breast Cancer; Real-time PCR.

Received: 14 October 2011

Revised accepted: 6 June 2012

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INTRODUCTION

Cancer is one of the highest causes of mortality in the world. The disease is attributed to various causes including mutagenesis and carcinogenic chemicals in the environment. Breast cancer accounts for 10.4 % of all cancer types among women; it is the most common type of non-skin cancer in women and the fifth most common cause of cancer death [1].

The high morbidity and mortality associated with breast cancer derive from its metastasis to lungs, bone and liver [2]. Metastasis is the major cause of death in human cancer patients and involves several stages, including loss of intracellular adhesion in the primary tumor region, migration into lymphatic or blood vessels, adhesion to the surface of the luminal endothelium, and invasion of other organ tissues [3].

Cisplatin (cis-diamminedichloroplatinum or cis-DDP/CDDP) is an anti-cancer drug widely used in the treatment of various cancers, including breast, testicular, ovarian, cervical, prostate, head and neck, bladder, and lung cancers [4]. Cisplatin can cause DNA damage by forming drug-DNA adducts and lead to apoptosis and/or necrosis. It, however, can also bind to other cell components such as glutathione, phospholipids, phosphatidylserine, microfilaments, thiol-containing proteins or RNA to cause cell damage. Cisplatin-induced cell death has been linked with ceramide-, mitochondria- and death receptor-mediated apoptosis, depending on the cell type being tested [5].

The *KAI1* gene encodes an integral membrane protein that consists of four transmembrane domains and one large extracellular domain, which indicates that *KAI1* protein is a member of the transmembrane 4 super family (TM4SF), also known as the tetraspanin superfamily [6]. *KAI1* is involved in cell migration, adhesion and synapse formation [7]. Many reports

have documented that the *KAI1* gene suppresses metastasis in many types of human cancers including breast, pancreatic, lung, bladder, hepatic, gastric, breast, colorectal, ovarian, esophageal, cervical and endometrial [8,9].

The aim of the current study was to investigate the effect of cisplatin on cell toxicity and metastasis through modulation of *KAI1* gene expression.

EXPERIMENTAL

Cell culture and cisplatin treatment

MCF-7, a human breast adenocarcinoma cell line was obtained from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (NCBI, C135). MCF-7 cells were 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 % CO₂). The cells were incubated with different concentrations of cisplatin (0, 10, 20, 40, 80 and 100 μM) at 24 h. Each concentration of cisplatin was tested on 3 wells of the 96-well plates containing 1×10⁴ MCF-7 cells. In each experiment, three MCF-7 cultured wells with no drug incubation were used as negative controls [10].

MTT assay and LC₅₀ determination

MTT solution (10 μl, 5 mg/ml in phosphate buffered saline, PBS) was added to the cell monolayer in each well of a 96-well plate. The cells were incubated in a humidified incubator at 37 °C for 3 h. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), into a dark blue formazan product which is insoluble in water. The insoluble formazan was dissolved in a solution containing 100 μl isopropanol and its optical density (OD) was read against a blank reagent with an ELISA reader at a wavelength of 570 nm. The 50 % lethal

concentration (LC_{50}) value of cisplatin on MCF-7 cells at 24 h was calculated. LC_{50} was determined by probit analysis using Pharm PCS (Pharmacologic Calculation System) statistical package (Springer Verlag, USA) [10].

Total RNA extraction

MCF-7 cells were rinsed with cold PBS. Thereafter, 2 ml of trizol (Invitrogen, USA) was added and incubated at room temperature for 5 min to lyse the cells in a culture dish. Afterward, 400 μ l of chloroform was added and the mixture incubated at room temperature for 3 min. The mixture was centrifuged at 12000 g (4 °C) for 15 min. The upper phase was transferred to a fresh Eppendorf tube and 500 μ l of isopropanol added to the mixture. After incubation on ice for 10 min, the sample was centrifuged at 12000 g (4 °C) for 15 min. Ethanol (75 %, 70 μ l) was added to the RNA pellets and the mixture was centrifuged at 7500 g (4 °C) for 5 min. The RNA pellets were dissolved in diethyl pyro carbonate (DEPC)-treated water. Finally, the concentration and purity of the isolated RNA were measured using a photonanometer (IMPLEN, Germany) at 230, 260 and 280 nm. RNA samples with the A260/A230 and A260/A280 ratios greater than 1.7 were selected for cDNA synthesis.

cDNA synthesis

cDNA synthesis was performed using 1st strand cDNA synthesis kit (Roche, Germany) in 20 μ l reaction mixture containing 3 ml of total RNA (1 μ g), 2 μ l reaction buffer 10x, 4 μ l $MgCl_2$ (25mM), 2 μ l deoxynucleotide mix (1mM), 2 μ l oligo-p(dT)15 primer (0.04 A_{260} units, 1.6 μ g), 1 μ l RNase inhibitor (50 units), 0.8 μ l AMV reverse transcriptase (20 units) and 7.2 μ l distilled water. Thermal cycling was performed in the ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) for 10 min at 25 °C, for 60 min at 42 °C (cDNA synthesis) followed by 5 min at 95 °C (reverse transcriptase inactivation) and then cooled to 4 °C for 5 min.

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 (Biorad Gel Doc XR, UK) and photographed. Gel electrophoresis (Payapajohesh Electrophoresis, Iran) was carried out to confirm the primers' specificity and amplification of PCR products.

Primer design

In this study, *KAI1* gene located on chromosome 11 (11p11.2) and *TBP* gene located on chromosome 6 (6q27), were selected as target and reference genes, respectively. Primers were designed using primer express software, v.3.0 (Applied Biosystems, USA). Synthesis of these primers was performed by Bioneer, South Korea. The sequence of the PCR forward primer for *KAI1* gene was 5' GTCACATGCTCATGGGCTTCC3' and that of reverse primer was 5' GAGGATCAGGAGCAGGAAAGC3'. The sequence of the forward primer for housekeeping gene *TBP* was 5' AATCATGAGGATAAGAGAGCCACG3' and that of reverse primer was 5' AGTCTGGACTGTTCTTCACTCTTGG3'. Primer specificity was tested using BLAST program (<http://www.ncbi.nlm.nih.gov/blast>).

Real-time-PCR

Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) was employed to perform quantitative PCR on ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), 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 15 s and 60 °C for 1 min. Each complete amplification stage was followed by a melting stage; at 95 °C for 15 s,

60 °C for 30 s and 95 °C for 15 s. PCR amplification was performed in 25 µl reaction mixture containing 12.5 µl Power SYBR Green PCR Master Mix (2x), 1 µl Forward primer (0.4µM), 1 µl Reverse primer (0.4µM), 5 µl first-strand cDNA (100 ng) and 5.5 µl double-distilled 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 37.5, 75, 150, 300 and 600 ng/µl of standard cDNA used. It was used to determine the dynamic range of the target and reference genes, to calculate the slope (PCR efficiency), R² (correlation coefficient), precision (standard deviation) and sensitivity (y-intercept). The efficiency of the reaction was calculated using Eq 1 [11].

$$E = [10^{(-1/\text{slope})} - 1] \dots\dots\dots (1)$$

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. Thereafter, mCt value of reference gene (*TBP*) was subtracted from mCt value of the target gene (*KAI1* gene) to obtain ΔCt and ΔΔCt values of each sample were calculated from corresponding Ct values; where ΔΔCt = [mCt target - mCt reference]_(untreated sample) - [mCt target - mCt reference]_(treated sample). Finally, *KAI1* gene expression/*TBP* gene expression ratio was calculated using Eq 2 [12].

$$\text{Ratio} = 2^{-\Delta\Delta C_t} \dots\dots\dots (2)$$

Statistical analysis

Data are expressed as mean ± standard deviation, correlation coefficients (R²) and assay reproducibility, and were processed using Microsoft Office Excel 2007 software. *P-value* of < 0.05 was considered statistically significant and this was assessed using Student's *t*-test.

RESULTS

Cisplatin cytotoxicity on MCF-7cells

Various concentrations of cisplatin (0, 10, 20, 50 and 100µM) at 24 h were cytotoxic to breast cancer cells (MCF-7cell line). At concentrations of 10, 20, 40, 80 and 100 µM of cisplatin MCF-7cell viability was reduced to 84.0 ± 12.4 (statistically insignificant, *p* > 0.05), 67.2 ± 12.4 (*p* < 0.05), 35.6 ± 3.4 (*p* < 0.001) and 30.7 ± 2.5 % (*p* < 0.001), respectively. LC₅₀ of cisplatin after 24 h was 143.4 ± 14.6 µg/ml, as reported in our previous study [10].

Absolute quantification analysis and PCR efficiency

We used different concentrations of cDNA for *KAI1* and *TBP* genes to prepare standard curves. The dynamic ranges of *KAI1* and *TBP* genes were obtained from 75 to 600 ng/µl. The slope of the standard curves were -3.31 (*KAI1*) and -3.36 (*TBP*). PCR efficiency was 92.30 % for *KAI1* and 98.15 % for *TBP* (Figure 1).

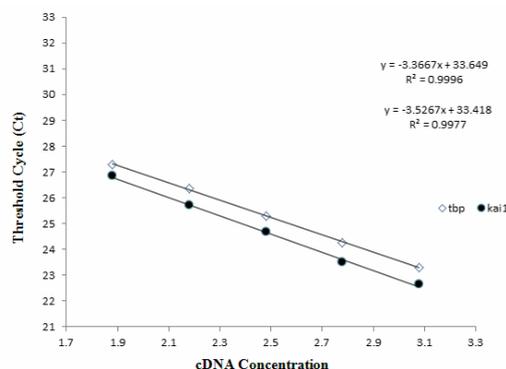


Figure 1: Standard curves for TBP (◇) and KAI1 (●) genes. Standard curve was generated by plotting Ct values against the logarithm of the cDNA concentration; for TBP, slope = -3.36, y-intercept = 33.649, R²= 0.999.;for KAI1, slope = -3.52, y-intercept = 33.418, R²= 0.997

Melting curve analysis and gel electrophoresis

The melting curve was drawn based on the temperature (x-axis) and ΔRn derivation (y-axis). The reproducibility of a melting curve

was high with a standard deviation of only 0.1°C between runs. It was generated to screen for primer dimers and to document single product formation for each gene. The melting peaks were drawn at 78.8 °C for *TBP* gene and 80.3 °C for *KAI1* gene as shown in Figure 2. Gel electrophoresis results showed specific amplification sequence of interest (Figure 3).

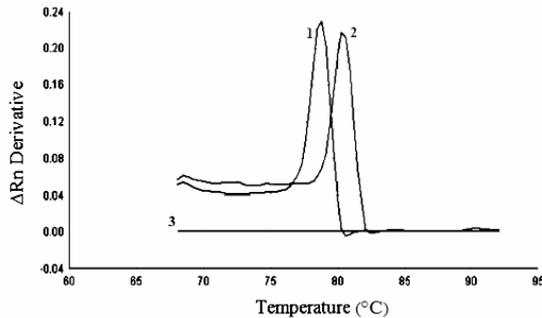


Figure 2: Specific melting curve analysis for TBP and KAI1 genes. The melting peaks at 78.8 °C for TBP gene (1) and 80.3 °C for KAI1 gene (2) indicate that the specific products melted at different temperatures. Flat peak demonstrates non-template control (3).

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 23.8 at different concentrations of cisplatin (0, 10, 20, 50 and 100µM). The values of mCts for *KAI1* gene were 33.1, 32.2, 31.6, 30.0 and 29.5 at 0, 10, 20, 50 and 100 µM concentrations of cisplatin, respectively. The mΔCt value for untreated sample was scaled as 9.30. The mΔCt values for treated samples of 10, 20, 50 and 100 µM of cisplatin concentrations were scaled as 8.32, 7.73, 6.12 and 5.67. The ΔΔCt values were scaled as -0.98, -1.57, -3.18 and -3.63 for 10, 20, 50 and 100 µM of cisplatin concentrations. The calculated $2^{-\Delta\Delta Ct}$ values were 1.97 ± 0.19 ($p < 0.05$), 2.96 ± 0.55 ($p < 0.05$), 9.06 ± 0.27 ($p < 0.001$) and

12.38 ± 0.88 ($p < 0.01$) for 10, 20, 50 and 100 µM of cisplatin concentrations (Figure 4).

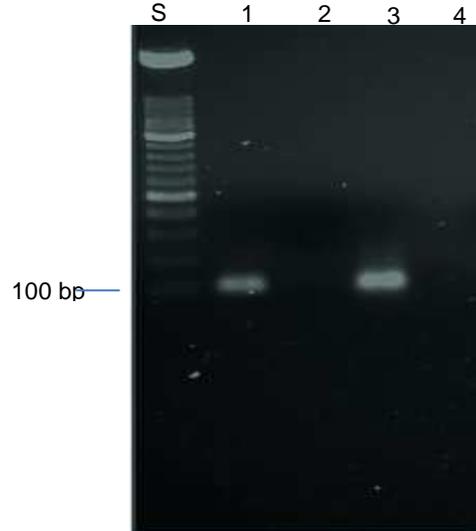


Figure 3: Photograph of a 1.5 % agarose gel showing TBP and KAI1 PCR products. Lane SM: = 100 bp size marker; Lane 1 = 96 bp PCR product of TBP gene; Lane 2 = non template control; Lane 3 = 100 bp PCR product of KAI1 gene; Lane 4 = non-template control.

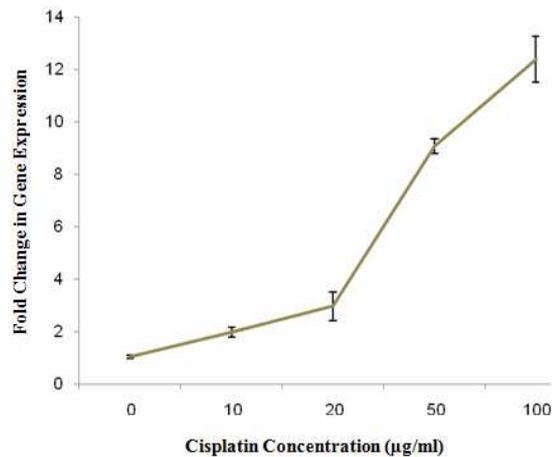


Figure 4: Fold change in KAI1 gene expression. Note: Cisplatin imposed an increase in mRNA level of KAI1 gene in a concentration-dependent fashion

DISCUSSION

The development of cancer is multiphasic, multigenic and multifactorial. Therefore, the anticancer efficacy of an agent will more likely depend on the nature and number of cellular, biochemical and molecular events being modulated by the agent as well as its side effects.

The most common sites of breast cancer metastasis are the lungs, bones, and liver [13]. Cisplatin is a first-line therapy for metastatic breast cancer. Cisplatin and gemcitabine have single-agent activity in metastatic breast cancer, and preclinical data support synergy of the combination [14]. Cisplatin is one of the major chemotherapeutic weapons used against different human cancers, although its mechanism of apoptosis induction is not fully understood.

Most cancer deaths are due to the development of metastasis, hence the most important improvements in morbidity and mortality will result from prevention of such disseminated disease. Metastasis has been found to be accompanied by various physiological alterations involved in the degradation of extracellular matrix (ECM), which allow cancer cells to invade blood or lymphatic system to spread to other tissues or organs. Tumor cell invasion and metastasis is associated with down-regulation of metastasis suppressor genes, the loss of function of which is an important event during the progression of a tumor cell from a non-metastatic to metastatic phenotype [15]. Several laboratories have identified more than 20 metastasis suppressors that inhibit metastasis without blocking tumor formation [16]. *KAI1* protein was first identified as a metastasis suppressor in prostate cancer. It has been documented that progression of metastasis is associated with down-regulation of *KAI1* [17]. Down-regulation of *KAI1* gene in advanced cancers does not appear to involve mutations [18]. *KAI1*, like other TM4SF proteins, has

been reported to interact with several integrins [19]. This association likely plays an important role in the function of *KAI1*-mediated metastasis suppression [6]. *KAI1* acts as a modulator of the integrin downstream signals and down-regulates the formation of the p130^{CAS}-Crk complex, leading to inhibition of cell migration [20]. We observed that cisplatin increased the expression of *KAI1* gene in MCF-7 cells at 24 h. These observations indicate that cisplatin can probably inhibit metastasis in breast cancer by inhibition of proliferation and increased expression of *KAI1* gene in MCF-7 cells.

CONCLUSION

For the first time, we showed in the present study that treatment with cisplatin for 24 h induces inhibition of proliferation of highly metastatic MCF-7 cells in a dose-dependent manner. Also, up-regulation of *KAI1* mRNA levels were observed in MCF-7 cells, in a dose-dependent manner. These findings indicate that cisplatin probably inhibits metastasis in breast cancer by inhibition of proliferation and increased expression of *KAI1* gene in MCF-7 cells. The results presented here warrant further investigation in animal tumor models.

ACKNOWLEDGEMENT

This work was funded by Pilot Biotechnology Department, Pasteur Institute of Iran, Tehran, Iran.

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