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Association between circadian gene *CLOCK* and cisplatin resistance in ovarian cancer cells: A preliminary study

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Abstract. The present study aimed to observe the expression of circadian gene clock circadian regulator (*CLOCK*) in ovarian cancer cells and the effects of circadian gene *CLOCK* on cis-dichlorodiamine platinum (cisplatin) resistance in ovarian cancer cells. The expression of *CLOCK* mRNA and protein in cisplatin-sensitive A2780 and cisplatin-resistant CP70 cells were detected by quantitative polymerase chain reaction and western blot assay. Cisplatin-sensitive A2780 and cisplatin-resistant CP70 cells were treated with different concentrations of cisplatin for 48 h, and the expression of hCLOCK protein in the two types of cells was detected by western blot assay. RNA interference method was used to knock down the expression of CLOCK in cisplatin-resistant CP70 cells. Subsequently, the cisplatin-resistant CP70 cells were treated with cisplatin. The proliferation of cisplatin-resistant CP70 cells was observed following treatment with cisplatin. The expression of *CLOCK* mRNA was significantly higher in cisplatin-resistant CP70 cells (1.58±0.49) compared with cisplatin-sensitive A2780 cells (0.44±0.13) ($P<0.01$). Western blot assay results demonstrated that the expression of CLOCK protein was significantly greater in the cisplatin-resistant CP70 cells (1.47±0.34) compared with the cisplatin-sensitive A2780 cells (0.48±0.15) ($P<0.01$). Following the treatment of A2780 and CP70 cells with cisplatin, CLOCK protein expression increased with an increased concentration of cisplatin, in a dose-dependent manner ($P<0.01$). Following the knockdown of CLOCK in cisplatin-resistant CP70 cells by RNA interference, cisplatin treatment was able to significantly inhibit the proliferation of cells and induce apoptosis ($P<0.01$). The expression of circadian gene *CLOCK* in ovarian cancer cells was strongly associated with cisplatin resistance. The upregulation of circadian gene *CLOCK* in ovarian cancer cells may reduce its sensitivity to cisplatin treatment.

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Key words: circadian gene, ovarian cancer, resistance, cisplatin, chemotherapy

Introduction

Ovarian cancer is one of the most common types of cancer in women, and the American cancer society estimated there would be approximately 22,000 new cases of ovarian cancer and 14,000 mortalities caused by the disease in 2017 in the United States, since ovarian cancer causes more deaths than any other cancer of the female reproductive system (1). The most common ovarian cancer types are known as epithelial ovarian cancer, which have the highest mortality rate amongst gynecological malignant tumors in females (2). More than 70% of patients with ovarian cancer have been found in advanced stage (International Federation of Gynecology and Obstetrics, FIGO stage III or IV) (3,4). Cis-dichlorodiamine platinum (cisplatin) is the main treatment for advanced ovarian cancer. However, the majority of patients with cisplatin resistance cannot benefit from chemotherapy (5). A variety of factors are involved in the emergence of cisplatin resistance, including increased drug efflux, abnormal drug targeting, enhanced DNA repair and altered apoptotic pathway (6-11). The basic mechanism for the emergence of chemotherapeutic drug resistance remains poorly understood. At present, there is a lack of effective drugs to reduce resistance to chemotherapy. Circadian genes are important genes, which regulate biological activity and includes period circadian clock (*PER)1*, *PER2*, *PER3*, timeless circadian clock (*TIM)*, clock circadian regulator (*CLOCK*), brain and muscle Arnt-like protein (*BMAL)*, cryptochrome circadian clock 1 (*CRY)1* and *CRY2* (11). Of these genes, *CLOCK* gene was the first gene identified, and has been revealed to be strongly associated with sensitivity of various tumor types to chemotherapy, including gastric cancer, cholangiocarcinoma and colorectal cancer (12-15). There are a limited number of studies that have investigated the association between the *CLOCK* gene and resistance to chemotherapy in ovarian cancer.

The present study observed *CLOCK* gene expression in cisplatin-sensitive cell line A2780 and cisplatin-resistant cell line CP70.

The effects of cisplatin on the proliferation and apoptosis of cisplatin-resistant cell line CP70 following the knockdown of *CLOCK* in cisplatin-resistant CP70 cells were investigated, and the effects of *CLOCK* on chemotherapy resistance in ovarian cancer were discussed.

Materials and methods

Cells and main reagents. Cisplatin-sensitive A2780 and cisplatin-resistant CP70 cell lines (Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China) were incubated in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Rat anti-human CLOCK antibody and horseradish peroxidase-labeled goat anti-rat immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA. Quantitative polymerase chain reaction (qPCR) primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). CytoBuster protein extraction reagent was purchased from Novagen (Merck KGaA, Darmstadt, Germany), and cisplatin was obtained from Sigma-Aldrich (Merck KGaA). Additionally, small-interfering (si)CLOCK small RNA fragments (Ambion; Thermo Fisher Scientific, Inc.), cell transfection reagent (Lonza Group, Ltd., Basel, Switzerland) and protease inhibitor phenylmethanesulfony fluoride (Thermo Fisher Scientific, Inc.) were also used. Phenylmethanesulfony fluoride was added to PBS prior to use.

Reverse transcription (RT)-qPCR. Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific). cDNAs were synthesized using a transcriptor first strand cDNA synthesis kit (Roche Applied Science, Penzberg, Germany), according to the manufacturer's protocol. mRNA levels were measured using using a FastStart Universal SYBR Green Master kit (Roche Applied Science) in a Light Cycler 96 (Roche Applied Science, Penzberg, Germany). Primer sequences were as follows: *CLOCK* forward, 5'-ACACCCAGAAGGAAGAGCAA-3' reverse, 5'-GCGAGAACGCTTTGCTTTAG-3'; *GAPDH* forward, 5'-ATGTCGTGGAGTCTACTGGC-3', reverse 5'-AGGATGCATTGCTGACAATC-3'. Reaction conditions are as follows: Pre-denaturation at 94˚C for 5 min, 40 cycles of 94˚C for 30 sec, 60˚C for 40 sec and 72˚C for 40 sec, followed by 72˚C for 10 min. Target gene fragments were amplified with a DNA thermal cycler. GAPDH was used as an internal reference. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method (16). Data were calculated from three independent experiments.

Cell treatment. Cisplatin-sensitive A2780 and cisplatin-resistant cell line CP70 cells ($1x10^5$/ml) (Shanghai Gefan Biotechnology Co., Ltd., Shanghai, China) were treated with 0, 32 and 64 μM dipeptidyl peptidase-4 (Exalpha Biologicals, Inc., Shirley, MA, USA) for 48 h at 37˚C.

Western blot assay. Total protein was extracted from cisplatin-sensitive A2780 and cisplatin-resistant CP70 cells in the logarithmic phase. Cisplatin-treated cells were digested with 0.25% trypsin, and total protein was extracted using CytoBuster protein extraction reagent. An equal volume of the protein (30 μg) was loaded onto 8% SDS-PAGE separating gel and 5% stacking gel and transferred to nitrocellulose membranes by semi-dry method. The membranes were blocked with Tris-buffered saline and Tween-20 containing 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 2 h at room temperature, and incubated with rat anti-human CLOCK antibody (1:1,500; cat no. 3896-100; BioVision, Inc., Milpitas, CA, USA) at 4˚C overnight. On the following day, the membranes were washed three times with 0.1% Tris-buffered saline and Tween-20 for 5 min each, incubated with horseradish peroxidase-labeled IgG secondary antibody at room temperature for 1 h and washed three times with 0.1% Tris-buffered saline and Tween-20. The membranes were incubated in SuperSignal West Pico substrate (Thermo Fisher Scientific, Inc.) for visualization, and were incubated with β-actin at 55˚C for 20 min, densitometry of the western blotting was analyzed with Image Pro Plus 6.0 (cat no. 20910; Media Cybernetics, Inc., Rockville, MD, USA). β-actin (1:1,500; cat no. MAB8929; R&D Systems, Inc., Minneapolis, MN, USA), served as the internal reference. The experiments were conducted at least in triplicate.

Cell transfection. The cell transfection reagent as mentioned previously was used. The cells were transfected with 100 nM of siCLOCK small RNA fragments for 48 h according to the manufacturer's protocol. At 24 h after transfection, an MTT assay was performed.

MTT assay. Following the knockdown of CLOCK protein expression, cisplatin-resistant CP70 cells at a density of $1x10^5$/ml and control cells (cells without gene knockout) at a density of $1x10^5$/ml were incubated in a 96-well plate at 37˚C. A total of 20 μl MTT was added in each well for 4 h at 37˚C. Following removal of the supernatant, 150 μl dimethyl sulfoxide was added, followed by a low oscillation speed for 10 min. Optical density was measured at 490 nm.

Statistical analysis. All data were analyzed with SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). t-test was used to compare the differences in data between two groups. One-way analysis of variance was utilized to compare the differences among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CLOCK mRNA expression in two types of ovarian cancer cells. As shown in Fig. 1, the expression of *CLOCK* mRNA was significantly higher in cisplatin-resistant CP70 cells (1.58±0.49) compared with cisplatin-sensitive A2780 cells (0.44±0.13) ($P<0.01$).

CLOCK protein expression in two types of ovarian cancer cells. As exhibited in Fig. 2, the expression of CLOCK protein was significantly higher in cisplatin-resistant CP70 cells (1.47±0.34) compared with cisplatin-sensitive A2780 cells (0.48±0.15) ($P<0.01$).

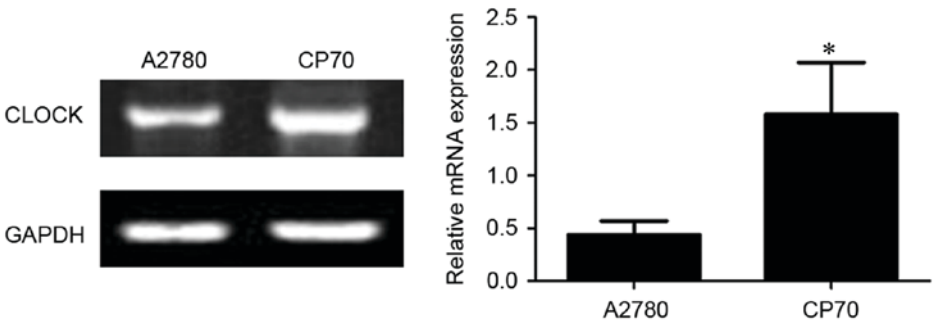

Figure 1. Quantitative polymerase chain reaction for *CLOCK* mRNA expression in ovarian cancer tissue. CLOCK, clock circadian regulator. *P<0.01 vs. cisplatin-sensitive A2780 cells.

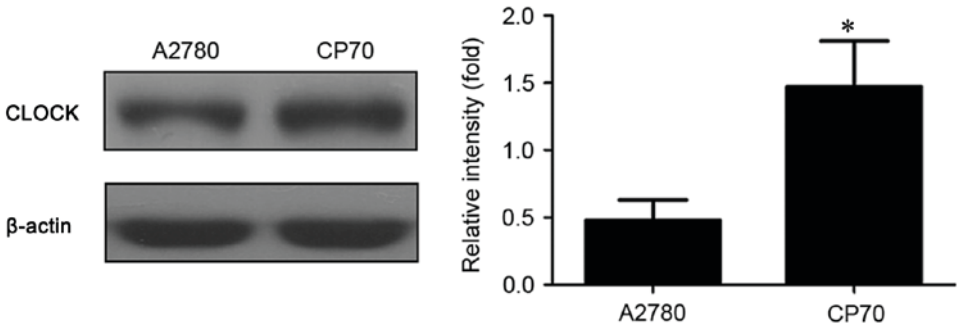

Figure 2. Western blot assay of CLOCK protein expression in A2780 and CP70 ovarian cancer cells. CLOCK, clock circadian regulator. *P<0.01 vs. cisplatin-sensitive A2780 cells.

Effects of cisplatin on CLOCK protein expression in two types of ovarian cancer cells. CLOCK protein expression was significantly increased in cisplatin-treated A2780 and CP70 cells compared with untreated cells (P<0.01; Fig. 3). Furthermore, CLOCK protein expression gradually increased with an increased concentration of cisplatin (P<0.01).

Confirming the effects of CLOCK knockdown on protein expression. As illustrated in Fig. 4, *CLOCK* siRNA transfection was able to significantly knockdown CLOCK protein expression in cisplatin-resistant CP70 cells compared with control cells (P<0.01).

Proliferation and apoptosis of cisplatin-resistant CP70 cells following CLOCK knockdown. As shown in Fig. 5, treatment with cisplatin did not significantly affect the proliferation and apoptosis of cisplatin-treated CP70 cells (P>0.05). By contrast following CLOCK knockdown, treatment with cisplatin was able to significantly inhibit the proliferation of CP70 cells and induce its apoptosis (P<0.01).

Discussion

Ovarian cancer is the fourth most common malignant tumor in women and is the leading cause of mortality from gynecologic malignancies. Due to its high mortality rate, ovarian cancer has become a global public health problem (17-20). The overall 5-year survival rate in the United States is 45%, the 1-year survival rate is 72% and the 10-year survival rate is 35% (21). For cases where a diagnosis is made early in the disease, when the cancer is still confined to the primary site, the 5-year survival rate is 92.7% (22). Approximately 70% of women with the advanced disease respond to initial treatment, a majority of whom attain complete remission, but half of these women experience recurrence 1-4 years following treatment (23). Therefore, if ovarian cancer can be diagnosed at an early stage, patients can get better treatment. Nevertheless, >70% of the patients with ovarian cancer have been diagnosed at advanced stages (FIGO stage III or IV) (4). The main method for advanced ovarian cancer is cisplatin treatment. However, the emergence of cisplatin resistance in the majority of patients with ovarian cancer reduces the effects of the chemotherapeutics (5).

A variety of different factors are involved in the emergence of cisplatin resistance, including increased drug efflux, abnormal drug targeting, enhanced DNA repair and altered apoptotic pathway (6-11). Molecular mechanisms of drug resistance in cisplatin-based chemotherapy remain unclear. There is a lack of effective drugs to reduce the resistance to chemotherapy.

Circadian rhythm is an endogenous adaptation mechanism in the process of long-term biological evolution and a basic characteristic of life activity. Circadian genes are important genes, which regulate biological activity and include *PER1*, *PER2*, *PER3*, *TIM*, *CLOCK*, *BMAL*, *CRY1* and *CRY2* (11). The *CLOCK* gene was the first gene discovered and was identified as a circadian clock gene. *CLOCK* gene is located on the long arm of chromosome 4 (4p12) and contains >20 exons (24). The *CLOCK* gene is not only expressed in normal tissue and cells. Abnormal expression of *CLOCK* gene can also be detected in

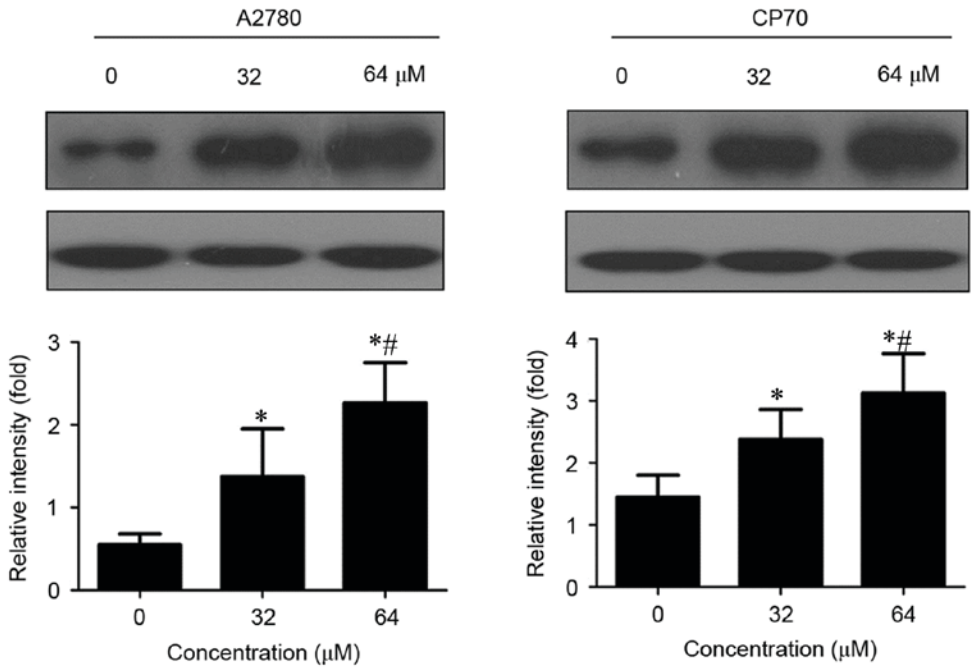

Figure 3. Effects of cisplatin on CLOCK protein expression in A2780 and CP70 cells. CLOCK, clock circadian regulator. *P<0.01 vs. untreated cells. #P<0.01 vs. 32 μM cisplatin-treated cells.

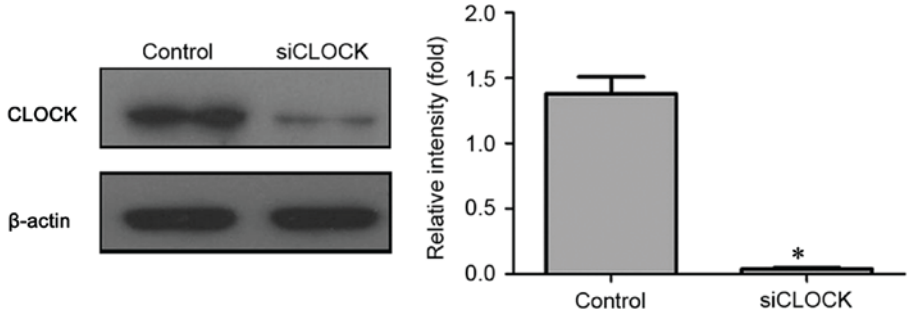

Figure 4. Western blot assay of CLOCK protein expression following CLOCK knockdown. CLOCK, clock circadian regulator; si, small-interfering. *P<0.01 vs. control cells.

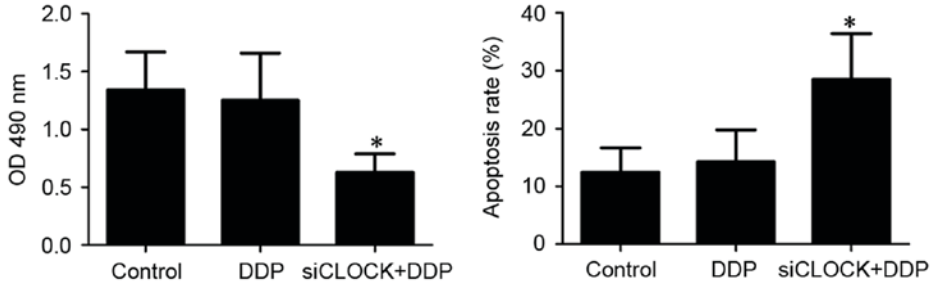

Figure 5. Proliferation and apoptosis of CP70 cells following CLOCK knockdown, as detected by MTT assay. DDP, cisplatin. *P<0.01, vs. control cells. CLOCK, clock circadian regulator.

a number of types of tumors (25-27). It was also demonstrated that the knockdown of CLOCK was able to increase the apoptosis of glioma cells (28). *CLOCK* gene has been revealed to be strongly associated with sensitivity to chemotherapy in various tumors, including gastric cancer, cholangiocarcinoma and colorectal cancer (13-15).

However, there are a limited number of studies that have investigated the association between *CLOCK* gene and chemotherapy resistance in ovarian cancer. Therefore, the present study observed *CLOCK* gene expression in cisplatin-sensitive A2780 and cisplatin-resistant CP70 cells and investigated the effects of cisplatin treatment on the

proliferation and apoptosis of cisplatin-resistant CP70 cells following *CLOCK* knockdown. The effects of *CLOCK* gene on chemotherapy resistance in ovarian cancer were also discussed.

The present study first compared *CLOCK* mRNA and protein expression in two ovarian cancer cell lines (A2780 and CP70). Results demonstrated that *CLOCK* mRNA and protein expression was significantly lower in cisplatin-sensitive A2780 cells compared with cisplatin-resistant CP70 cells ($P<0.01$), indicating that *CLOCK* gene expression was strongly associated with cisplatin resistance in ovarian cancer cells. To further verify the association between *CLOCK* gene and cisplatin resistance in ovarian cancer cells, cisplatin-sensitive A2780 and cisplatin-resistant CP70 cells were treated with different concentrations of cisplatin. Results showed that CLOCK protein expression increased with an increased concentration of cisplatin in the two cell lines following cisplatin treatment in a dose-dependent manner ($P<0.01$), which further suggested that *CLOCK* gene was associated with cisplatin resistance in ovarian cancer cells. To confirm the precise effect of *CLOCK* gene on cisplatin resistance in ovarian cancer cells, the present study knocked down the expression of CLOCK protein in cisplatin-resistant CP70 cells by RNA interference. The results showed that cisplatin treatment was able to significantly suppress the proliferation of CP70 cells, and induce its apoptosis following the knockdown of CLOCK protein ($P<0.01$). In summary, the expression of circadian gene *CLOCK* was strongly associated with cisplatin resistance in ovarian cancer cells. The increase in the expression of circadian gene *CLOCK* may reduce the sensitivity to cisplatin treatment in ovarian cancer cells.

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Availability of data and materials

All relevant data are included in the present study.

Authors' contributions

HX conceived and designed the experiments, and wrote the manuscript. ZW, GM and HC conducted the experiments, collected, analyzed and interpreted the data. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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