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ORIGINAL ARTICLE

Low-dose fractionated radiation reverses cisplatin resistance in ovarian cancer cells via PI3K/AKT/GSK-3β signaling

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Abstract

Objective To investigate whether low-dose fractionated radiation (LDFRT) could enhance cisplatin sensitivity in drug-resistant human ovarian cancer cells SKOV3/DDP, and to further explore the underlying mechanism.

Methods SKOV3/DDP ovarian cancer cells were divided into three groups as follows: control, LDFRT, and conventional-dose radiation groups. Cells from all three groups were treated with different concentrations of cisplatin (0, 1.25, 2.5, 5, 10, and 20 μg/mL) for 48 h. The proliferation inhibition rate was investigated using the cell counting kit 8 (CCK8). The rate of apoptosis was determined by flow cytometry (FCM). Protein levels of AKT, P-AKT, GSK-3β, P-GSK-3β, P21, cyclin D1, and P27 were examined by Western blotting.

Results As expected, LDFRT significantly reduced the half-maximal inhibitory concentration (IC_{50}) of cisplatin and promoted apoptosis in SKOV3/DDP cells. Moreover, in the LDFRT group, protein levels of P-AKT, P-GSK-3β, and cyclin D1 were markedly decreased, those of P21 and P27 were greatly increased, and total AKT and GSK-3β levels showed no significant difference compared to those in both the control and conventional-dose radiation groups.

Conclusion LDFRT sensitizes resistant SKOV3/DDP ovarian cancer cells to cisplatin through inactivation of PI3K/AKT/GSK-3β signaling.

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Key words: low-dose fractionated radiation (LDFRT); cisplatin-resistance; ovarian cancer; PI3K/AKT/GSK-3β pathway

Ovarian cancer (OC) is the most lethal gynecological malignancy and is the fifth primary cause of mortality from cancer among women worldwide [1]. Currently, the standard therapy for OC is cytoreductive surgery combined with platinum (carboplatin or cisplatin)-based chemotherapy. Although chemotherapy could achieve an initial effective rate of 80%, 85% of patients with advanced OC experience relapse and succumb to the disease at a median of 18–24 months after diagnosis because of acquired resistance to the currently available chemotherapeutic drugs [2–3]. Thus, chemo-resistance remains the first problem to consider in this disease. Exploring the molecular mechanisms of drug resistance and searching for novel treatments is thus necessary for patients with OC [4].

Considering the limitations of chemotherapy for OC, radiation has attracted a wide spectrum of people. Unfortunately, non-irradiated cells appear to suffer many side effects including DNA double strand breaks, sister chromatid exchange, genomic instability as bystander effects, and micronucleus formation [5] because of the radiation. Consequently, a new treatment model combining full-dose chemotherapy with low-dose fractionated radiation (LDFRT) without causing significant toxic and side effects is emerging6-7. Joiner *et al* found that low doses of radiation (LDR) (< 100 cGy) show a hyper-radio sensitivity (HRS) phenomenon in the initial phase, at which cells die from hypersensitivity to small single doses (1–80 cGy) of radiation. Additionally, HRS does

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not activate cell-repair mechanisms, which explains why there is no induction of radiation-resistance with HRS *in vitro*. Four fractions of LDR that were reduced to 50–100 cGy indicated the maximal cell death in mitotic cells and the least tissue damage in senescent cells when combined with chemotherapy *in vitro*. Shareef *et al* found that compared with higher and conventional doses of chemoradiotherapy, LDFRT enhances the efficacy of chemotherapy without resistance development [8–9].

PI3K/AKT, as an elementary survival and anti-apoptotic signaling pathway, plays a pivotal role in many cellular processes including survival, proliferation, metastasis, motility, and drug resistance in multiple tumor types [10–11]. Generally, mutations or amplification of PIK3CA (encoding the catalytic PI3K P110α subunit), abnormal AKT expression, and PTEN deletion are the proverbial ways that activate the signaling pathway in about 70% of OC [12]. Depending on membrane localization, the AKT family could be phosphorylated at the Thr-308 and Ser-473 sites. Therefore, Ser-473 phosphorylation represents AKT activity [13]. Importantly, phosphorylated AKT (P-AKT) is capable of suppressing apoptosis by inactivating many pro-apoptotic factors including the serine (Ser)/threonine (Thr) kinases glycogen synthase kinase-3 (GSK-3)α and GSK-3β [14–15].

GSK-3β is a functional Ser/Thr kinase that plays an essential role in the modulation of glycogen metabolism, cell differentiation, embryonic development, and cell apoptosis [16]. GSK-3β activity can be promoted by phosphorylation at tyrosine (Tyr-216), but can be lowered by phosphorylation of Ser-9 [17]. GSK-3β induces apoptosis by stimulating downstream pro-apoptotic factors as its regulatory proteins, while its inactive status can downregulate pro-apoptotic effects and suppress cell death induction through phosphorylation [18]. Nevertheless, whether GSK-3β has the ability to promote or suppress tumors remains controversial [19–20]. There is increasing evidence that GSK-3β activity regulates the efficacy of chemotherapeutic drugs on tumor cells. Recently Tian Wang et al found that increased GSK-3 expression is responsible for paclitaxel resistance in OC cells [21]. Downregulation of GSK-3β has been one of the major factors fostering cisplatin resistance in OC cells [22].

In this study, we examined the cellular response to the conventional dose of radiation (200 cGy) and LDFRT (200 cGy delivered in four fractions) combined with different doses of cisplatin. Furthermore, the influence of PI3K/AKT/GSK-3β signaling on the cisplatin-resistant human OC cell line, SKOV3/DDP, was investigated and the antitumor mechanisms of LDFRT were explored.

Materials and methods

Cell culture and reagents

Cisplatin-resistant human OC cells, SKOV3/DDP, were obtained from the Institute of Cancer Research, Chinese Academy of Medical Sciences. (Beijing, China). Cells were cultivated in Roswell Park Memorial Institute 1640 (RPMI-1640) (HyClone Ltd, China) medium supplemented with 10% fetal bovine serum (FBS; Gibco Co., Australia) at 37°C in a humidified incubator with 5% CO_2. To maintain drug resistance, the cells were additionally cultured with cisplatin (Qilu Pharmacy Co. Ltd, China) at a concentration of 0.5 μg/mL. The culture medium was changed every 2–3 days according to the cellular quorum. Cell Counting Kit and Annexin V-FITC/PI Apoptosis Detection Kit were purchased from Dojindo (Dojindo Laboratories, Japan). Antibodies against AKT, GSK-3β, P27, P21, and cyclin D1 were purchased from Bioss (Bioss Biotech Co. Ltd., China). Antibodies against phospho-Akt [p-Akt (Ser473)] and phospho-GSK-3β [(p-GSK-3β (Ser9)] were purchased from EnoGene (EnoGene Biotech Co., Ltd, China). Secondary antibodies conjugated to horseradish peroxidase, and the protease inhibitor were from Odyssey (Odyssey Biotech Co. Ltd., China).

Radiation conditions

The Varian 23EX Electron linear accelerator and 6 MV X-ray were used to radiate the cells. The area of the radiation field was 10 × 15 cm, and the source skin distance (SSD) of the cells was 100 cm. The dosage rate was 300 cGy/min. The cells were randomly divided into three groups as follows: LDFRT, conventional-dose, and control groups. Cells in the LDFRT group were exposed to 50 cGy radiation each time (total dose 200 cGy in four fractions every 8 h). Cells in the conventional-dose group were subjected to 200 cGy radiation at one time, whereas the control group was given no radiation. All cells were then treated with different concentrations of cisplatin.

Cell viability assay

The CCK8 assay was performed to determine cell viability. Cells in the logarithmic growth phase were seeded in 96-well plates at a density of 5×10^3 cells/well and cultured for 24 h. The culture medium was then replaced with medium containing various concentrations of cisplatin (0, 1.25, 2.5, 5, 10, and 20 μg/mL). We also set up a blank group containing neither drugs nor cells. Cisplatin-treated cells were incubated for 72 h, and 10 μL of CCK-8 solution was then added to each well for an additional 3 h. The optical density (OD) was measured at 450 nm, and cell proliferation was determined. The survival rate of cells (%) = (Experimental group OD value – Blank group OD value) / (Control group OD value –

Blank group OD value), and the rate of cell proliferation inhibition (%) equaled 100% minus the survival rate. The half-maximal inhibitory concentration (IC_{50}) was calculated by the weighted linear regression method using SPSS 21.0 software. Each experiment was repeated thrice.

Cell apoptosis analysis

Annexin V/Propidium iodide (PI) staining for flow cytometry (FCM) was used to quantify DDP-induced apoptosis. Cells from the three groups were incubated in 6-well plates at a density of 1×10^6 cells/well, and cultured with different concentrations of cisplatin (0, 1.25, 2.5, 5, 10, and 20 μg/mL) for 48 h. The cells were then harvested, centrifuged for 10 min at 1500 rpm, and washed twice with ice-cold PBS. The cells were then resuspended in 500 μL of binding buffer and sequentially stained with 5 μL of Annexin V-FITC and 5 μL of PI solution for 15 min in the dark, as per the manufacturer's instructions. Apoptosis rate was determined by flow cytometry (BD FACSCalibur system). The percentages of early apoptotic cells (high FITC and low PI signal) and the late apoptotic cells (high FITC and high PI signal) were plotted and analyzed using FlowJo software.

Western blot analysis

SKOV3/DDP cells were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation (RIPA) buffer (Beyotime Institute of Biotechnology, China) for protein extraction followed by centrifugation at 12,000 ×g for 20 min at 4°C. The protein concentration was assayed using the BCA Protein assay kit (Beyotime Institute of Biotechnology Jiangsu, China), and the samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels followed by transfer to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in Tris-buffered saline with Tween (TBST) (in mmol/L: NaCl 150, Tris-HCl 20, pH 7.5, 0.05% Tween 20) containing 5% skim milk for 1.5 h at room temperature. The membranes were then incubated overnight at 4°C with the relevant primary antibodies (against AKT, P-AKT, GSK-3β, P-GSK-3β, P21, cyclin D1, P27, and GAPDH) (1:500), followed by appropriate secondary antibodies conjugated with horseradish peroxidase (1:1000). The proteins were visualized using the enhanced chemiluminescence (ECL) Western blotting system, and ImageJ software was used to analyze the bands.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software. All the values from the groups were each expressed as mean ± standard deviation (SD). Statistical differences between two groups were compared using the Student's *t*-test and among multiple groups by one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

Results

Effect of LDFRT on SKOV3/DDP cell proliferation

To determine whether LDFRT has an inhibitory effect on SKOV3/DDP cell viability, we performed the CCK8 assay. SKOV3/DDP cells from all three groups were exposed to cisplatin at concentrations of 0, 1.25, 2.5, 5, 10, and 20 μg/ml for 48 h. Cells from all three groups were found to be suppressed in a dose-dependent manner, but cells from the LDFRT group showed higher inhibition rates than those in the other two groups (Fig. 1). Cisplatin cytotoxicity at different concentrations was evaluated by IC_{50} to demonstrate the change in cell sensitivity to cisplatin after simultaneous treatment with LDFRT. The results showed that cells from the LDFRT group were more sensitive to cisplatin induced cytotoxicity compared with cells from other groups (Fig. 1).

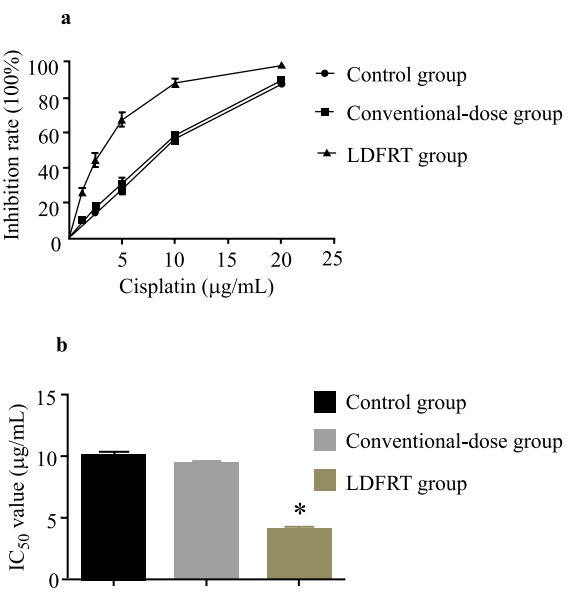

Fig. 1 SKOV3/DDP cell inhibition rates and IC_{50} in three groups. Error bars represent mean ± SD. $P < 0.05$, * $P < 0.05$ versus control group and conventional-dose group

Effects of LDFRT on SKOV3/DDP cell inhibition rates and IC_{50} in three groups

LDFRT inhibited the survival of SKOV3/DDP cells. SKOV3/DDP cell viability was examined by the CCK8 assay at different concentrations of cisplatin as indicated. LDFRT showed higher inhibition rates (Fig. 1; $P < 0.05$) and lowered IC_{50} value (Fig. 1; $P < 0.05$) of SKOV3/DDP cells compared to those of the conventional-dose and control groups.

Effects of LDFRT on SKOV3/DDP cell apoptosis

After treatment of SKOV3/DDP cells from the three groups with different concentrations of cisplatin, apoptosis induction by cisplatin was examined by Annexin V-FITC and PI staining using a flow cytometer (Fig. 2). The total apoptosis rate (early apoptosis rate plus late apoptosis) in the LDFRT group was significantly increased compared with that in the conventional-dose and control groups (Fig. 2; $P < 0.05$), suggesting that LDFRT can significantly enhance apoptosis in cisplatin-resistant OC cells *in vitro.*

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Effects of LDFRT on the apoptosis of cisplatin-resistant SKOV3/DDP cells, demonstrated with FCM

Cells were treated with different concentrations of cisplatin after exposure to different doses of radiation, and were then tested using FCM. The results are indicated as the percentage of predominantly late apoptotic or dead cells in the first quadrant: Annexin-FITC (+) and PI (+), predominantly necrotic and dead cells in the second quadrant: Annexin-FITC (–) and PI (+), non-apoptotic and viable cells in the third quadrant: Annexin-FITC (–) and PI (–), and early apoptotic cells in the fourth quadrant: Annexin-FITC (+) and PI (–) (Fig. 2). The total apoptosis (the first and fourth quadrants) was examined.

Influence of LDFRT on PI3K/AKT/GSK-3β signaling-related proteins in SKOV3/DDP cells

The PI3K/AKT/GSK-3β pathway and its downstream proteins in SKOV3/DDP cells were further examined by Western blot analysis. As shown in Fig. 3, the expression levels of P-AKT, P-GSK-3β, and cyclin D1 were markedly decreased in the LDFRT group ($P < 0.05$) whereas the expression levels of P21 and P27 proteins

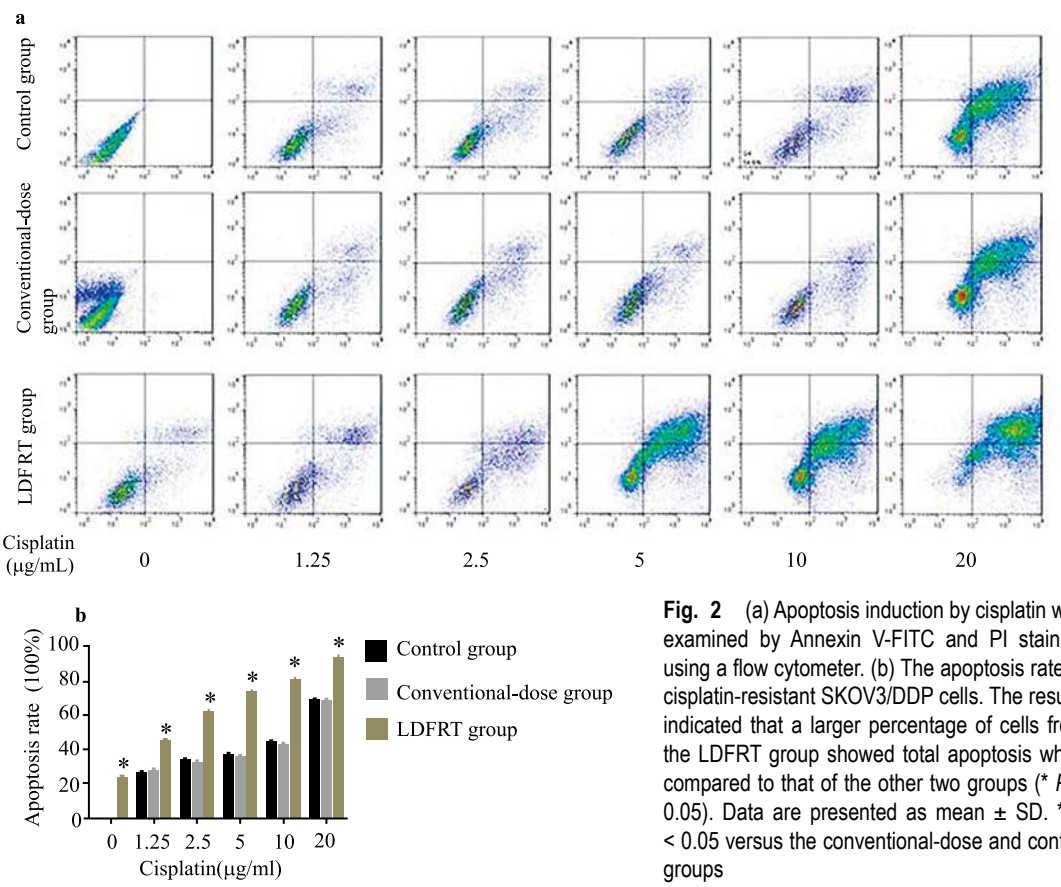

Fig. 2 (a) Apoptosis induction by cisplatin was examined by Annexin V-FITC and PI staining using a flow cytometer. (b) The apoptosis rate of cisplatin-resistant SKOV3/DDP cells. The results indicated that a larger percentage of cells from the LDFRT group showed total apoptosis when compared to that of the other two groups (* $P < 0.05$). Data are presented as mean ± SD. * $P < 0.05$ versus the conventional-dose and control groups

were significantly increased (P < 0.05) compared to those in the conventional-dose and control groups. However, total AKT and GSK 3β expression showed no significant difference among all three groups. Taken together, the data showed that LDFRT plays a potential role in improving sensitivity to cisplatin by inhibiting the activity of the PI3K/AKT/GSK 3β pathway. This suggests a strong correlation between PI3K/AKT/GSK 3β signaling and cisplatin resistance in OC cells.

Western blot analysis of PI3K/AKT/GSK-3β-related protein expression in SKOV3/DDP cells

GAPDH was used as the internal control in this analysis. As demonstrated in Fig. 3, the levels of P-AKT, P-GSK-3β, and cyclin D1 were significantly reduced (P < 0.05) whereas the levels of P21 and P27 were significantly increased (P < 0.05) in the LDFRT group compared to those in the conventional-dose and control groups. However, the total AKT and GSK-3β levels showed no significant differences among the three groups.

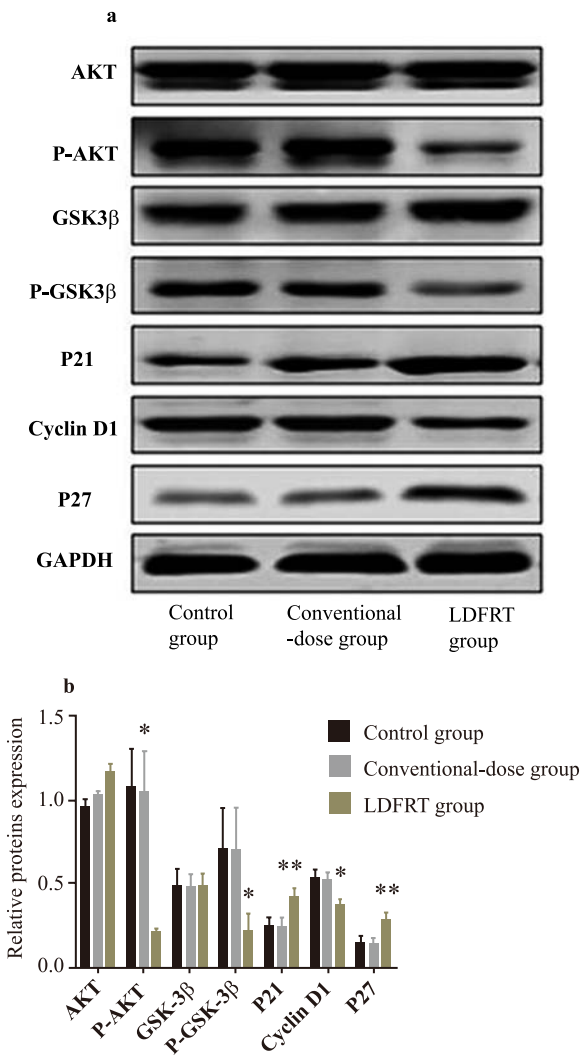

Fig. 3 Western blot analysis of PI3K/AKT/GSK-3β-related protein expression in SKOV3/DDP cells. Data are presented as mean ± SD. * P < 0.05 and ** P < 0.05 versus control group and conventional-dose group

Discussion

OC is considered as the primary cause of death among all gynecological cancers worldwide as its 5-year survival rate remains < 30% [23]. The major obstacle in effective OC therapy is the development of resistance against the first-line treatment, which results in relapse within 3 years after chemotherapy among more than 80% of patients with advanced OC [24]. The mechanism underlying cisplatin resistance is still not completely clear.

When combined with LDFRT, the therapeutic effect of chemotherapy could be enhanced, and a study by Kunos et al. demonstrated that LDFRT was well tolerated and improved the chemosensitivity of docetaxel in patients with recurrent OC, without increasing the side effects of docetaxel [6, 8]. In addition, previous studies by our team found that LDFRT stimulated the growth of normal cells but not tumor cells *in vitro* and *in vivo*, and could reverse cisplatin resistance in OC by suppressing DNA damage repair and promoting apoptosis through the ERCC1 and Bcl-2 gene [25]. In the present study, our data demonstrated that compared with those of the conventional-dose radiation and control groups, the cells of the low-dose radiation group showed a higher rate of proliferation inhibition and apoptosis. Moreover, as assessed by Western blot analysis, there was no difference in total AKT and GSK 3β protein levels as previously reported. However, their inactivating forms, P-AKT and P-GSK-3β, were significantly decreased. LDFRT also induced cell cycle arrest, which was accompanied by increased P27 and P21 expression and decreased cyclin D1 expression [26]. The cellular response to DNA damage includes cell cycle delays, increased cellular repair, and apoptosis [27]. DNA damage causes cell cycle arrest in the G1, S, or G2/M phases to prevent replication of damaged DNA or to prevent aberrant mitosis. Studies have shown that AKT is usually activated in cancer cells, and its activation (P-AKT) promotes cell proliferation and suppresses apoptosis [28]. A recent study by Zhang *et al* demonstrated that AKT plays a major role in controlling cell cycle progression and indicated that inhibition of AKT could overcome the cell cycle checkpoint induced by DNA damage and promote apoptosis [26]. GSK-3β is a multifunctional enzyme related to many cellular processes such as chemoresistance, cell growth, survival, apoptosis, and metastasis [29]. The role of GSK-3β in cancer development remains controversial. Farago *et al* have indicated that GSK-3β promotes tumorigenesis, whereas Ding *et al* have shown that GSK-3β leads to tumor

suppression and chemosensitization [30–31]. Consistent with the latter, we found that GSK-3β is necessary for LDFRT-induced apoptosis. GSK-3β inhibits cell proliferation and enhances apoptosis by downregulating its downstream proteins such as P21, P27, and cyclin D1 [32–33]. P21 and P27 are proverbial cell cycle inhibitors. P21 can suppress cell cycle progression and modulate apoptosis after DNA damage and P27 plays an important role in modulating cell proliferation [34–35]. Cyclin D1, as one of the G1 cyclins and a major regulator of the G1 restriction point, is usually found in high-grade OC. Overexpression of cyclin D1 was found to be positively correlated with reduced cancer cell survival and chemosensitivity [36].

Consequently, the PI3K/AKT/GSK-3β pathway plays an important role in cisplatin-resistance in patients with OC and LDFRT may enhance the cellular susceptibility to cisplatin by suppressing this pathway.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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