Journal of Oncology Research and Treatments

Zhang et al., J Oncol Res Treat 2020, 5:2

Research Article Open Access

Metformin Sensitizes Ovarian Cancer Cells to Chemotherapy By Inhibiting ERCC1 Expression

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Abstract

Metformin is an oral anti-hyperglycemic agent of the biguanide family, which is used first-line for type II diabetes with few side-effects. Clinical studies demonstrated that diabetics taking metformin significantly decreased incidence of cancer, which has opened the prologue of metformin in cancer research. Since recent studies have revealed the inhibition of cancer cells' growth and remission time prolonged effect of the anti-diabetic reagent metformin but the underlying mechanisms of ovarian cancer remain unclear, we investigated the potential effects and possible molecular mechanisms of metformin on chemosensitivity of ovarian cancer cells. Cell proliferation were determined with combination of cisplatin and metformin by MTT and ERCC1 expression level were analyzed by Western blotting assay. In addition, ERCC1 plasmid transfection was used to explore ERCC1 roles in the synergistic effect of cisplatin and metformin on ovarian cancer cells. The results indicated that treatment with metformin enhanced the sensitivity of ovarian cells to cisplatin and cisplatin induced ERCC1 expression could be suppressed by metformin. Furthermore, over-expression of ERCC1 in ovarian cancer cells could abrogate the synergistic effect of cisplatin and metformin. In conclusion, metformin enhanced the sensitivity of ovarian cancer cells to cisplatin by down-regulation the expression of ERCC1 and may be a prospective chemotherapeutic agent or a chemosensitizer in future ovarian cancer treatment.

Keywords: Metformin; Cisplain; ERCC1; Chemosensitivity; Ovarian cancer

Introduction

Ovarian cancer, one of the most common malignant tumors in gynecology, seriously threatened global women's health. In China, the proportion of patients suffering from ovarian cancer among various types of cancer were very high, and the mortality rate of patients was about 21.6% [1]. After tumor resection, platinum-based chemotherapy was the most effective treatment for ovarian cancer at this stage [2]. Although the initial surgical treatment and chemical treatment were highly effective, recurrence, metastasis and chemotherapy resistance even leading to poor prognosis often occurred after treatment [3]. There have been few new progresses in the treatment of patients with ovarian cancer since the standardization of platinum-based chemotherapy [4]. Therefore, for ovarian cancer patients who are resistant to chemotherapy, more effective treatment strategies are necessary.

Metformin is widely used to treat type II diabetes obesity and polycystic ovarian syndrome [5]. In addition to its own anti-diabetic properties, metformin has been recently considered to be used for anticancer therapy [6]. Studies have confirmed the anti-proliferation effect on various human cancer cell types such as breast, stomach, prostate, liver, and could significantly enhanced the effect of cisplatin on breast, lung and cervical cancer in nude mice [7-12]. Moreover, metformin has been found to improve the chemotherapy response in diabetic patients with ovarian cancer [13]. However, the mechanism behind this process has not been evaluated.

One of the key enzymes in the Nucleotide Excision Repair (NER) pathway is Excision Repair Cross Complementarity 1 (ERCC1), which was involved in DNA damage identification and DNA strand cleavage [14]. Several studies have been indicated that ERCC1 levels were correlated with resistance to platinum-based chemotherapy both in cellular and clinical and were induced by cisplatin to repair DNA damage and enhance drug resistance [15-17]. It is suggested that ERCC1 could be a potential molecular target in cancer chemotherapy as down-regulation of ERCC1 expression can increase responses to cisplatin treatment.

In this study, we examined whether metformin can increase the sensitivity of ovarian cancer cells to chemotherapy and to explore the role of ERCC1 in this process. In conclusion, metformin enhanced the sensitivity of ovarian cancer cells to cisplatin by down-regulation the expression of ERCC1 and may be a prospective chemotherapeutic agent or a chemosensitizer in future ovarian cancer treatment.

Materials and Methods

Cell lines and reagents

Human ovarian cancer A2780 lines taken from the American Type Culture Collection (ATCC) were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) complete medium, add 10% Fetal Bovine Serum (FBS). SK-OV-3 cell was donated by Professor Pixu Liu (Dalian Medial University). The rabbit anti-ERCC1 and anti-β-actin human polyclonal antibodies purchased from The Protein Technology Group, lnc (Chicago, USA). Metformin and cisplatin were purchased from Sigma (St.Louis, MO, USA).

Cell viability assays: The effect of metformin and cisplatin on the viability of A2780 and SK-OV-3 cells was detected by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT,

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Received March 19, 2020; **Accepted** June 19, 2020; **Published** June 26, 2020

Citation: Zhang C, Li X, Li H, Liang Y, Zhang J, et al. (2020) Metformin Sensitizes Ovarian Cancer Cells to Chemotherapy By Inhibiting ERCC1 Expression. J Oncol Res Treat 5: 147.

Sigma, St.Louis, MO, USA) assay. The cells (1×105/well) were seeded into 96-well plate and incubated for 24 h. Then medium containing cisplatin (0, 2, 4, 6, 8, 16, 24, 32, 64 μM) or combined with 10 μM metformin were added into each well. After treating 48 h, 20 μl MTT was added to each well (5 mg/mL in PBS) incubated for 4 h. The MTT solution was removed and added into 15 μl Dimethyl sulfoxide (DMSO Sigma-Aldrich). The 96-well plate was assayed by a Multiskan Ascent plate reader (Thermo Fisher Scientific) at 540 nm wavelength.

Colony formation assay

Cells were plated at 500 cells per 100 mm dish 24 h before treatment. Then 500 ul medium containing with or did not contain cisplatin (2 μM) and metformin (1 μM) were added into each well. The cells were fixed with 70% ethanol and stained with crystal violet dissolved in 10% ethanol (Sigma, St. Louis, Missouri, USA) 15 days later. The number of colonies were obtained by counting and each colony were defined with at least 50 cells.

Construction and transfection of expression vector

A2780 reverse transcription of cDNA with transcriptional taq reverse transcriptase and random hexamer primer. The ERCC1-coding region was amplified by Polymerase Chain Reaction (PCR). The PCR product were cloned into mammalian prokaryotic expression vector pcDNA3.1.PcDNA3.1-ERCC1 or pcDNA3.1 vectors were transfected into A2780 cells with liposome 2000 transfection reagent (Invitgen, USA) as negative control.

Western blot analysis

The cells were collected and lysed in cold RIPA buffer. The protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membrane by semi-dry transfer apparatus for 40 mins. The membrane was sealed with 5% non-fat milk for 1 h and then incubated overnight with the specific primary antibody solution overnight at 4°C. Horseradish Peroxidase (HRP) labeled secondary antibody detection signal, ECL kit (TERMO, USA) to display the signal RNA isolation and Reverse Transcriptase-Quantitative Polymerase Chain Reaction (qRT-PCR) analysis.

Quantitative reverse transcription PCR (qPCR)

Total RNA was extracted from A2780 cells using Trizol regent (life technologies, California) according to the instructions given by the manufacturer. cDNA synthesized by cDNA synthesis super mix kit (TransGen Biotech, Beijing, China) was in 20 μl reaction volume. Then 1 μg of cDNA template was applied to amplify specific genes by the qRT-PCR assay using SYBR green master mix kit (Qiagen, Germany). qRT-PCR reaction was conducted in Applied Biosystems 7500 PCR instrument (Applied Biosystems, Carlsbad, CA).The forward primer sequence for ERCC1 was 5'-CTGGGAATTTGGCGACGTAA-3' and reverse primer sequences was 5'-ATGGATGTAGTCTGGGTGCAG-3'.

Statistical analysis

All experiments were independently repeated three times. The results a represented as average Standard Deviation (SD). Student's t-test was performed to compare groups using SPSS version 14.0 (SPSS, Chicago, IL, USA). The difference between the experimental group and the control group were evaluated by unpaired t-test. The value of $p<0.05$ were considered to be statistically significant.

Results

Metformin can enhance the sensitivity of ovarian cancer cells to chemotherapeutic drugs

To detect the efficacy of metformin combined with cisplatin, MTT assay were performed. The results showed that metformin significantly enhanced the inhibitory effect of cisplatin on the growth of ovarian cancer cells A2780 and SK-OV-3 compared with cisplatin alone for both treating 48 h. The synergistic effect of low dose cisplatin and metformin was the most significant ($P<0.01$) (Figure 1A and 1B).

Cisplatin increased ERCC1 expression in ovarian cancer cells

To investigate the role of ERCC1 in cisplatin resistance in ovarian cancer, we detected the level of ERCC1 protein after cisplatin treatment by Western blotting (Figure 2A and 2C). The results of ovarian cancer cells A2780 and SK-OV-3 demonstrated a dose-dependent increase in protein levels of ERCC1 ($P<0.05$) (Figure 2B and 2D). It is suggested

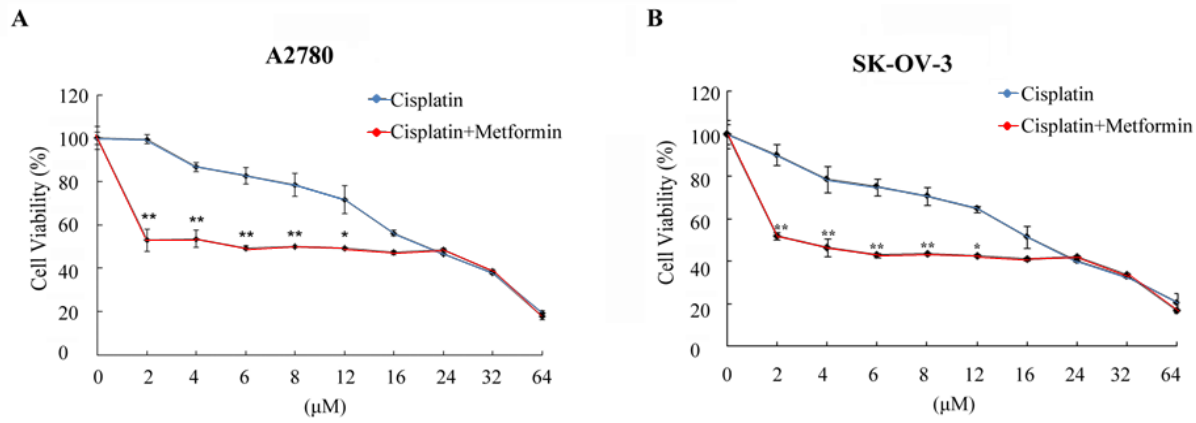

Figure 1: Synergistic enhanced cell cytotoxicity by combining cisplatin with metformin. (A) A2780 and (B) SK-OV-3 Cells treated with cisplatin (0, 2, 4, 6, 8, 16, 24, 32, 64 μM) or combined with 10 μM metformin for 48 h. The percentage of cell growth was determined by MTT assay. Metformin combined with cisplatin significantly inhibited cell growth compared to cisplatin alone. The results represent the means ± SD of triplicate samples. Three independent experiments were carried out. $p<0.05$; $^{**}p<0.01$, Single factor analysis of variance.

that cisplatin could induce ERCC1 expression by repairing DNA damage and leading to drug resistance.

Metformin reduced ERCC1 expression in ovarian cancer cells

We also detected the expression of ERCC1 after metformin treatment. Western blotting analysis demonstrated a dose-dependent decrease in the expression of ERCC1 protein after the effect of metformin both in A2780 and SK-OV-3 ovarian cancer cells (P<0.001) (Figure 3A-3D). We found that metformin could repress ERCC1 expression in ovarian cancer cells which assumed that the synergistic effect of metformin and cisplatin on ovarian cancer cells was due to ERCC1 expression change.

Combination treatment with cisplatin and metformin repressed ERCC1 expression

We further examined the expression of ERCC1 in the cells

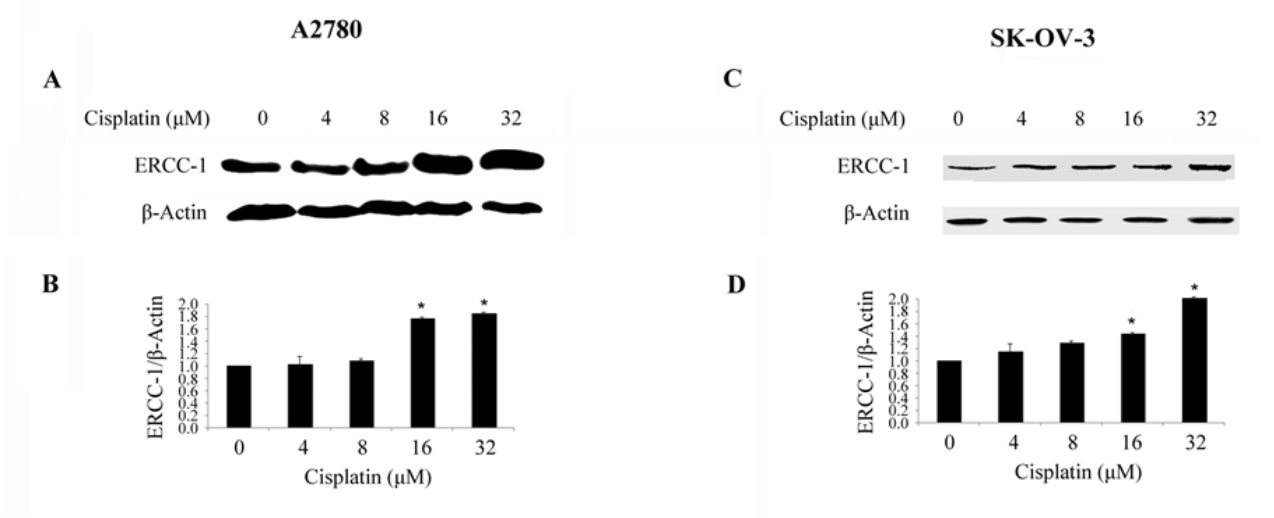

Figure 2: Cisplatin increased ERCC1 expression in ovarian cells. The cells were treated with cisplatin (0, 4, 8, 16, 32 μM) for 24 h. The whole cell extract was collected and analyzed by Western blotting. (A) Treatment of A2780 cells with cisplatin resulted in a dose-dependent increase of ERCC1 expression. (B) The relative expression of ERCC1 protein in A2780 cells were calculated based on β-actin expression. (C) Cisplatin significantly increased the expression of ERCC1 in SK-OV-3 cells. (D) The relative expression of ERCC1 protein in SK-OV-3 cells were calculated based on β-actin expression. Three independent experiments were performed. p<0.05; **p<0.01, one-way analysis of variance.

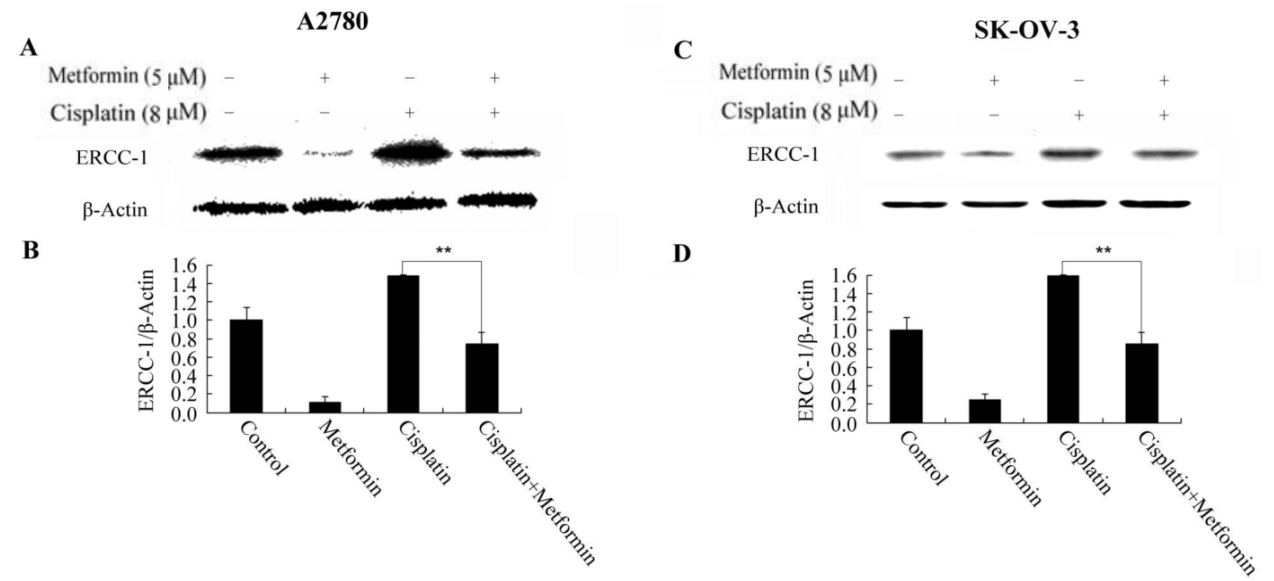

Figure 3: Metformin decreased ERCC1 expression in ovarian cells. The cells were treated with metformin (0, 5, 15, 25, 35,45 μM) for 24 h. Whole-cell extracts were collected for western blotting analysis. (A) Treatment of A2780 cells with metformin resulted in a dose-dependent reduction of ERCC1 expression. (B) The relative expression of ERCC1 protein in A2780 cells was calculated based on β-actin expression, which was used as loading control. (C) Metformin significantly decreased the expression of ERCC1 in SK-OV-3 cells. (D) The relative expression of ERCC1 protein in SK-OV-3 cells was calculated based on β-actin expression. Three independent experiments were performed. **p<0.05;***P<0.001, one-way analysis of variance.

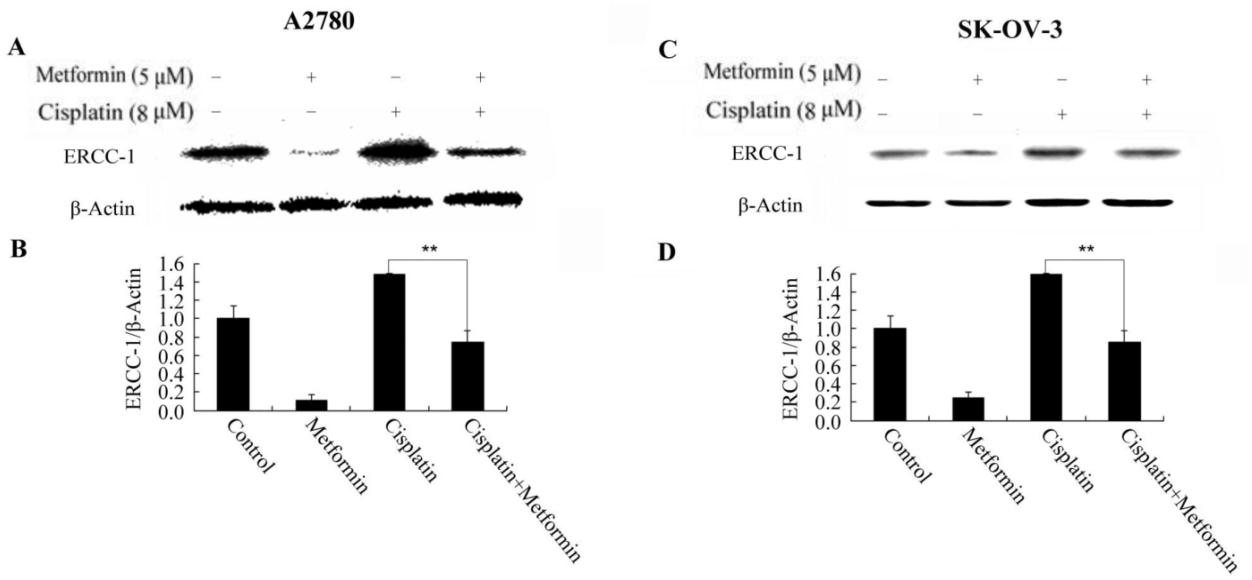

Figure 4: Combination treatment with cisplatin and metformin repressed ERCC1 expression. (A) A2780 cells were treated with metformin (5 μM), cisplatin (8 μM) or metformin (5 μM) together with cisplatin (8 μM) for 24 h. Collect whole-cell extract for Western blotting analysis. β-Actin served as loading control. (B) Histogram showed means ± SD of ERCC1 against level of β-actin. (C) SK-OV-3 cells were treated with metformin (5 μM), cisplatin (8 μM) or metformin (5 μM) together with cisplatin (8 μM) for 24 h. Whole-cell extracts were collected for Western blotting analysis. β-Actin served as loading control. (D) Histogram showed means ±SD of ERCC1 against level of β-Actin. Three independent experiments were performed. **P<0.05, one-way analysis of variance.

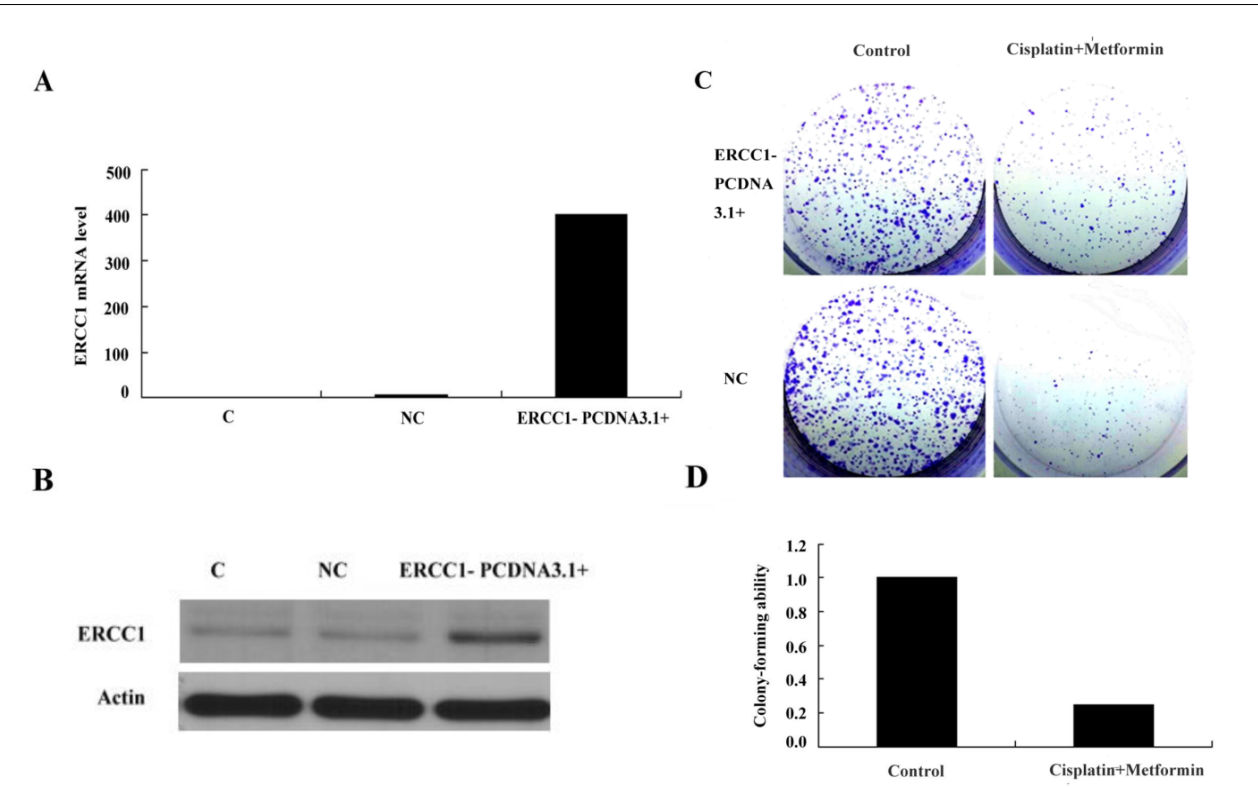

Figure 5: Overexpression of ERCC1 induced chemotherapeutic drug resistance. (A) The A2780 cells were transfected with either pcDNA3.1-ERCC1 or pcDNA3.1 vector as Negative Control (NC). qRT-PCR were used to detect the expression of ERCC1. Histogram showed means ± SD of ERCC1 against β-Actin. (B) Collect whole-cell extract of transfected cells and NC for Western blotting analysis. β-Actin served as loading control. (C-D) Overexpression of ERCC1 delayed colony formation after exposure to cisplatin together with metformin. The cells transfected with pcDNA3.1-ERCC1 formed more colonies than negative control cells. Three independent experiments were performed. **P<0.01, one-way analysis of variance.

exposure to cisplatin together with metformin by Western blotting to investigate whether metformin could restrain the induction of ERCC1 by cisplatin (Figure 4A and 4C). Consistent to our previous data, the expression of ERCC1 both in A2780 and SK-OV-3 ovarian cancer cells were increased in cisplatin treatment but decreased in metformin. Moreover, the induction of ERCC1 expression by cisplatin was significantly suppressed by metformin ($P<0.01$) (Figure 4B and 4D).

Overexpression ERCC1 induced the resistance to chemotherapeutic drugs

To explore the role of ERCC1 in combined therapy, the ovarian cancer cells were transfected with pcDNA3.1-ERCC1 plasmid and then treated with metformin and cisplatin. Cytotoxicity were detected by colony formation assay. The qRT-PCR data showed the expression of ERCC1 significantly increased in the transfected cells compared with the negative control cells ($P<0.001$) (Figure 5A). Overexpression of ERCC1 also plotted in the transfected cells compared with the negative control cells (Figure 5B). After exposure to cisplatin together with metformin, the ovarian cancer cells transected with pcDNA3.1-ERCC1 formed more colonies than the negative control cells ($P<0.01$) (Figure 5C and 5D).The results indicated that overexpression of ERCC1 could abrogate the synergistic effect between cisplatin and metformin.

Discussion

Ovarian cancer is a reproductive system tumor and a leading cause of death among female patients suffering from gynecological malignancy which threatening global women's health [18,19]. Chemotherapy is a common treatment strategy for patients, but most of these tumors are unfortunately not sensitive to chemotherapy and even develop resistance to these drugs, which is currently a major obstacle to the treatment of ovarian cancer [20]. Moreover, increasing the dose of chemotherapeutic drugs may cause side-effects on patients [21]. Therefore, at present, the most important problem is to find a reasonable strategy to reduce the dose of chemotherapeutic drugs and improve the sensitivity of cancer cells.

Various studies have reported the role of metformin in the treatment and prevention of cancer [22,23]. It has also been reported that metformin enhances the cytotoxicity of cisplatin *in vivo* to inhibit the growth of ovarian cancer [24]. Consistent to these studies, our data showed that metformin had inhibitory effects on the growth of ovarian cancer cells and could also increase the sensitivity of ovarian cancer cells to cisplatin therapy. Although cisplatin alone could inhibit cell proliferation in ovarian cancer, the toxic side-effects would increase with drug doses [25,26]. Our data showed that the toxic side-effective dose of cisplatin could greatly reduce in the chemotherapy combined with metformin.

We further investigated the molecularly mechanisms under this process. Previous study indicated that cisplatin could induce expression of DNA repair protein ERCC1 and enhance cisplatin resistance in melanoma cells [27]. In this search, our data also demonstrated that ERCC1 dose-dependent expression of ovarian cancer increased after cisplatin treatment. We suspected that metformin can increase the sensitivity of ovarian cancer cells to cisplatin by down-regulating ERCC1. Therefore, we detected the expression of ERCC1 after metformin treatment. The results showed that metformin indeed decreased the ERCC1 protein level. In addition, the induction of ERCC1 expression by cisplatin could be inhibited by metformin administration. Furthermore, overexpression of ERCC1 promotes colony formation of ovarian cancer cells induced by cisplatin and metformin. It suggested that the synergistic effect between cisplatin and metformin was due to down-regulation of ERCC1 expression by metformin.

Conclusion

In conclusion, metformin can enhance the sensitivity of ovarian cancer cells to chemotherapeutic drugs. Moreover, we elucidated that, for the first time, DNA repair gene ERCC1 involved in the synergistic effect of metformin and cisplatin on ovarian cancer cells. Therefore, the combination of metformin and cisplatin in the treatment of ovarian cancer may be a new therapeutic strategy and may be a prospective chemotherapeutic agent or a chemosensitizer in future ovarian cancer treatment.

Acknowledgements

This work was supported by The Chinese National Natural Science Fund (No. 81302282).

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