Succinate Dehydrogenase Subunit B Regulates Cisplatin Resistance via DNA Damage in Human Ovarian Cancer Cells

Lilan Chen

Department of Obstetrics and Gynecology, Renji Hospital, School of Medicine, Shanghai Jiaotong University

Wen Di (diwen163@163.com)

Department of Obstetrics and Gynecology, Renji Hospital, School of Medicine, Shanghai Jiaotong University

Research Article

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Abstract

Background

Succinate dehydrogenase subunit B (SDHB) is one of succinate dehydrogenase subunits which is an important enzyme in the tricarboxylic acid (TCA) cycle. SDHB has been found expressed low in human ovarian carcinoma compared to human ovarian normal epithelium tissues. Previous studies have confirmed that the high expression of SDHB significantly inhibits the proliferation, invasion and migration of ovarian cancer cells, indicating that SDHB plays a role of tumor suppressor gene in ovarian cancer. But the role of SDHB in ovarian cancer chemotherapy is unknown.

Methods

Using the SDHB specific siRNA and overexpression plasmid, the expression of SDHB was silenced and conversely overexpressed in ovarian cancer cell lines SKOV3 and A2780, respectively. The role of SDHB in ovarian cancer chemotherapy was investigated in vitro, using CCK8 and Colony formation assays. Immunohistochemistry was used to exam the expression of SDHB in human ovarian cancer cisplatin-resistant tissues and corresponding cisplatin-sensitive tissues. To explore the mechanism, DNA damage repair related proteins such as γ-H2AX and ERCC1were examined by western blot in SKOV3 and A2780 cells.

Results

SDHB expressed lower in human ovarian cancer cisplatin-resistant tissues than the corresponding cisplatin-sensitive tissues. *SDHB* silencing increased drug resistance and *SDHB* overexpression showed the opposite results in SKOV3 and A2780 cells. In *SDHB*-silenced cancer cells, the level of ERCC1, which contributes to platinum drug resistance by promoting DNA repair, was increased, andγ-H2AX, which is related to cell apoptosis and drug resistance was decreased.

Conclusions In this research, we demonstrated *SDHB* silencing reduced chemosensitivity in SKOV3 and A2780 by improving the repair of DNA damage. These results suggested that SDHB might represent a potential therapeutic target to overcome chemosensitivity in human ovarian carcinoma.

Background

Ovarian carcinoma is one of the leading causes of high mortality rate in gynecological malignant tumors. The reasons for this phenomenon are clinical symptoms of patients who have suffered from ovarian carcinoma are discovered in late stage. Furthermore, there are drug resistance and cancer recurrence during the therapy process[1]. In order to improve the drug treatment effect, researchers try their best to seek new ways for ovarian cancer therapy.

Succinate dehydrogenase (SDH), also known as succinate-ubiquinone oxidoreductase, is a mitochondrial enzyme complex that catalyzes the oxidation of succinate to fumarate in the citric acid cycle, it also

participates in the electron transport chain. There are several subunits (*A, B, C, D*) of this enzyme[2], it is known that mutations of the *SDH* gene determine the genetic basis of paragangliomas/pheochromocytomas[3, 4] and associative tumor syndromes (Carney-Stratakis syndrome and Carney triad)[5]. Meanwhile, Kusao I et al. reported that mRNA expression of *SDHB* decreased in recovering Ramos cells in childhood non-Hodgkin lymphoma (NHL)[6]. What's more, immunohistochemistry results showed that loss of SDHB expression was associated with an adverse outcome in pheochromocytomas/sympathetic paragangliomas (PCCs/PGLs). It was suggested SDHB protein loss as a marker of adverse outcome both in sporadic and in familial PCC/PGL[7]. We can conclude SDHB may play an important role in tumors. In this study, we aimed to gain a better understanding of the wider consequences of transient SDHB inactivation by creating SKOV3 and A2780 cell lines in which *SDHB* was silenced by RNAi. We found *SDHB*-silenced cells have lower ability of chemosensitivity to DDP. In order to study the role of SDHB deeply in human ovarian carcinoma, we designed *SDHB* overexpression plasmid *pIRES2-EGFP*. We found the opposite results which showed the important role of *SDHB* in SKOV3 and A2780 cells.

The DNA repair mechanism is a crucial molecular pathway potentially involved in resistance to platinum-based chemotherapy. Excision repair cross-complementing1 (ERCC1) plays a leading role in the nucleotide excision repair pathway, and a relationship between ERCC1 expression and platinum resistance has been reported in patients with ovarian[8, 9], gastric[10], bladder[11], and colorectal[12]. As a part of post-translational modifications during apoptosis caused by severe DNA damage, high expression of phosphorylated H2A.X (γ-H2AX) is considered as an accurate indicator of apoptosis[13] and it is also related to drug chemosensitivity[14]. In our research, *SDHB* affected repair of DNA damage related proteins in ovarian cancer cells. These findings implicate *SDHB* play an important role in ovarian cancer chemosensitivity. It is suggested that overexpression of *SDHB* may be an effective therapeutic strategy for treatment of ovarian cancer.

Methods

Tumor specimens and Ethics Statement

A total of 12 tissue specimens were collected from surgical patients enrolled in this study, including 6 human ovarian cancer cisplatin-resistant tissues and 6 corresponding cisplatin-sensitive tissues between July, 2011 to August, 2015. All specimens, obtained during surgery (Department of Obstetrics and Gynecology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, China), frozen immediately in liquid nitrogen and stored at − 80℃ until analysis. All carcinoma patients were diagnosed with ovarian tumors, and received chemical therapy after the surgery. The study was approved by the Institutional Review Board of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consents were obtained from all patients. Clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

Immunohistochemistry

The paraffin-embedded tissue samples were deparaffinized, rehydrated and placed into citric acid buffer (pH 6.0, 0.1 M) for heating for 10 min. The endogenous peroxidase activity was then blocked by incubation with 3% H2O2 for 10 min. Afterwards, sections were incubated with blocking buffer (Beyotime, China) for 1 h and then incubated overnight at 4°C with SDHB antibody (1:200, Epitomics). Following a 10 min incubation of biotinylated second antibody, the slides were again incubated with streptavidin-peroxidase under the same condition. The immunoreaction was then visualized by incubation with diaminobenzidine chromogen (DAB, Maixin-Bio, China) for 5 min. Finally, the slides were counterstained with hematoxylin, dehydrated, cleared and mounted. Negative controls were incubated in blocking buffer alone. These results were only considered if these control samples demonstrated a negative staining.

Cell lines and cell culture

The human epithelial ovarian adenocarcinoma cancer (EOC) cell lines SKOV3 and A2780 were obtained from Chinese Academy of Science (Shanghai, China) and cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. The medium was replaced every 24 h.

SiRNA transfection

SiRNA oligonucleotides were synthesized from Shanghai Integrated Biotech Solutions Co.,Ltd. The target sequences were as follows: siSucA *5′-GAT TAA GAA TGA AGT TGA CTC-3′*. As a control for silencing, we constructed a negative control (NC, NC-FAM) siRNA (*5′-UUC UCC GAA CGU GUC ACG UTT-3′*). Transient transfection was carried out according to the manufacturer's instructions of Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA).

Plasmid construction and transient transfection[15]

The full length *SDHB* opening reading frame was sub-cloned into *pIRES2-EGFP* plasmid. The plasmid was purchased from Shanghai Integrated Biotech Solutions Co., Ltd. SKOV3 cells were transfected with *pIRES2-EGFP SDHB* vector cDNA using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Vector-only transfectants cells were used as controls in the following experiments.

Real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA), then total RNA was reverse transcribed with a PrimeScript RT reagent Kit (TaKaRa, Shanghai, China). Resultant complementary DNAs (cDNAs) underwent quantitative real-time reverse transcriptase PCR using a SYBR Green PCR Master Mix Reagent Kit (TaKaRa) according to the manufacturer's protocol. Real-time PCR and data collection were performed on Step one real-time system (Applied Biosystems). The primers for *SDHB* were *5′- AAA TGT GGC CCC ATG GTA TTG-3′* (forward) and *5′-AGA GCC ACA GAT GCC TTC TCT G-3′* (reverse). The primers for *β-actin*, serving as the endogenous controls, were *5′-TGA CGT GGA CAT CCG CAA AG-3′* (forward) and *5′-CTG GAA GGT GGA CAG CGA GG-3′* (reverse). SDS v.1.4 (Applied Biosystems) software was used to perform comparative delta cycle threshold (Ct) analysis.

Western blot analysis

Cells were lysed on ice for 30 min using RIPA buffer and PMSF (Beyotime, Shanghai, China). Proteins were separated by SDS-PAGE and transferred to a Polyvinylidene Fluoride membrane (Millipore, Bedford, MA). After blocking with 5% dry milk in TBST for 2 h at room temperature, the membranes were incubated with the primary antibodies against human SDHB (1:1000, Epitomics), β-tubulin (1:4000, Epitomics), β-actin (1:500, Abmart), ERCC1 (1:400, Abcam), phospho-histone H2A.X (γ-H2A.X, 1:1000, Cell Signaling Technology) in dilution buffer overnight at 4°C. After washing three times with TBST, memmbranes were incubated with IRDye 800CW Conjugated Goat (polyclonal) Anti-Rabbit IgG or Anti-Mouse IgG (1:10000) antibodies for 1 h at room temperature. The expression of specific proteins was detected through the use of Odyssey system following the manufacturer's instructions.

Chemosensitivity analysis

Ovarian cancer cells (5×10^3/well) (48h post-transfection) were incubated with different concentrations of cisplatin (DDP; Qi Lu Pharmacy Co., Ltd, China) for 24h in 96-well plate. Cell viability was measured by the CCK8 assay kit (Dojindo, Japan). The absorbance was determined at 450 nm using FLx800 Fluorescence Microplate Reader (Biotek). The median inhibitory concentration (IC50) was analyzed.

Colony formation assay

Ovarian cancer cells were treated with indicated concentration of cisplatin (DDP; Sigma) for 12 h or 1 h after *SDHB* silencing or overexpressing for 48 h, then cells were plated in 6-well plates at a density of $1 \cdot 10^3$ cells per well under normal culture conditions for 8 days, the media was changed every 3 days. At the end, cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, photographed using a Nikon D80 digital camera, and colonies were counted.

Statistical analysis

All data were expressed as the means ± standard error (SEM). An independent Student t test was used to compare the continuous variables between groups. A P value less than 0.05 ($P < 0.05$) was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.0 software (San Diego, CA).

Results

SDHB expressed lower in human ovarian cancer cisplatin-resistant tissues than the corresponding cisplatin-sensitive tissues

Previously, it has been shown that SDHB is low expressed in ovarian cancer, and that overexpression of SDHB inhibit cell proliferation, invasion, and migration, indicating that SDHB may play an anti-oncogene role in ovarian cancer[15]. Thus far, the role of SDHB in cisplatin resistance of ovarian cancer has not been elucidated. To investigate the expression of SDHB in cisplatin sensitive or resistant ovarian cancer, ovarian cancer tissue samples were obtained from 6 women with recurrent epithelial ovarian cancer in 6 months after standard therapy, and other 6 women who were chemosensitive. All patients received

optimal cytoreductive surgery followed by 6 cycles of systemic chemotherapy with the combination of cisplatin and paclitaxel. All the slides were from the ovarian cancer tissues resected in the initial operation. The paraffin-embedded slides were immunehistochemically stained with SDHB antibody and representative stained slides were shown in Fig. 1A.These results indicated that cisplatin resistant ovarian cancer exhibit lower level of SDHB expression compared to cisplatin sensitive ovarian cancer (Fig. 1).

SDHB **silencing increased drug resistance in ovarian cancer cells**

Given that SDHB was expressed lower in human ovarian cancer cisplatin-resistant tissues, we further investigate the effect of SDHB on drug resistance of ovarian cancer cells. We transiently transfected siRNA targeting SDHB in SKOV3 and A2780 cells, SDHB mRNA and protein expression was greatly reduced (***$P < 0.001$) (Fig. 2A,2B). After 48 h transfection, cells were treated with DDP for 24 h and then CCK8 assay was used to examine cell viability. IC50 was upregulated in *SDHB*-inhibited SKOV3 and A2780 cells compared to the control after DDP treatment for 24 h (**$P < 0.01$, *$P < 0.05$) (Fig. 2C). Colony formation assay was performed at 12 h and 1 h post-cisplatin treatment respectively. Treatment with SDHB siRNA and DDP also resulted in significant increase in SKOV3 and A2780 cell numbers as measured by colony formation assay (*$P < 0.05$) (Fig. 2D).

SDHB **overexpression inhibited drug resistance in ovarian cancer cells**

SKOV3 cell line was chosen to overexpress SDHB. After treated with SDHB overexpressed plasmid for 24 h, SDHB mRNA level was up-regulated 33 fold in SKOV3. Under the same condition, SDHB protein expression was increased after 48 h transfection (***$P < 0.001$) (Fig. 3A). After 48 h transfection, cells were treated with DDP for 24 h and then CCK8 assay was used to examine cell viability. IC50 was downregulated in *SDHB* overexpressed SKOV3 cells compared to the control after DDP treatment for 24 h (**$P < 0.01$, *$P < 0.05$) (Fig. 3B). Colony formation assay was performed at 12 h and 1 h post-cisplatin treatment respectively. Treatment with SDHB overexpressed plasmid and DDP also resulted in significant decrease in SKOV3 cell numbers as measured by colony formation assay (*$P < 0.05$) (Fig. 3C).

SDHB **affected repair of DNA damage in ovarian cancer cells**

We found *SDHB* silencing could upregulate the median inhibitory concentration (IC50) of DDP and overexpression *SDHB* could decrease IC50 in SKOV3 and A2780 cells (Fig. 2C,3B). In *SDHB*-silenced cancer cells, the level of ERCC1, which contributes to platinum drug resistance by promoting DNA repair, was increased, and γ-H2AX, which is related to cell apoptosis and drug resistance was decreased (Fig. 4A,4B). *SDHB* overexpression cells showed the opposite results (Fig. 4C). Moreover, colony formation was partially promoted in *SDHB*-silenced SKOV3 cells after DDP treatment for 12 h (Fig. 2D), while *SDHB* overexpression cells showed the opposite results (Fig. 3C). These results showed *SDHB* silencing could reduce sensitivity of ovarian cancer cells to DDP.

Discussion

Ovarian carcinoma is the most lethal gynecological cancer which causes more deaths than any other gynecologic malignancies. In order to more effectively treat and prevent ovarian cancer, gynecological researchers face the challenge of developing a full understanding of the molecular events leading to initiation and progression of the disease, but there are limit for controlling cancer growth, metastasis and chemosensitivity. SDHB has been reported to be downregulated in several cancer types[3, 5] [16].

Consistent with these studies, we found that lower SDHB mRNA and protein were detectable in human ovarian carcinoma than normal ovarian surface epithelium. In addition, *SDHB* mRNA expression was lower in primary ovarian carcinoma than the corresponding metastasis. Previous studies have confirmed that the high expression of SDHB significantly inhibits the proliferation, invasion and migration of ovarian cancer cells, indicating that SDHB plays a role of tumor suppressor gene in ovarian cancer [15]. In order to further study the role of SDHB in ovarian cancer chemotherapy, we found that the expression of SDHB was low in drug-resistant tissues of ovarian cancer through immunohistochemical detection. Cytological test found that the low expression of SDHB significantly inhibited the cisplatin sensitivity of ovarian cancer and enhanced the clonogenic ability of ovarian cancer cells, but the specific mechanism was unknown.

The chemoresistance of ovarian cancer is closely related to DNA damage repair [17]. When the DNA damage repair ability of tumor cells is enhanced, which reduces the chemosensitivity of tumor cells, and then affects the development of tumor [18]. We also discovered *SDHB* knockdown could decrease SKOV3 and A2780 cell sensitivity to DDP in association with an increase in ERCC1 and an decrease in γ-H2AX. ERCC1 has been linked to platinum resistance in ovarian cancer[8, 9], non-small cell lung cancer[19, 20], and small cell lung cancer[21]. Moreover, time to progression and overall survival in EOC patients previously treated with platinum-based chemotherapy are significantly longer in patients with low ERCC1 expression compared with patients with high ERCC1 expression. In a retrospective study, high expression levels of ERCC1 were observed in 49 (44.1%) of 111 patients with small cell lung cancer who received front-line platinum-based chemotherapy[21]. The DNA double-strand break (DSB) is a serious lesion that can initiate genomic instability, ultimately leading to cancer[22, 23]. A key component in DNA repair is the histone protein H2AX, which becomes rapidly phosphorylated on a serine four residues from the carboxyl terminus (serine c-4) to form γ-H2AX at nascent DSB sites[24]. In addition to being a cause of cancer, DSB induction is paradoxically an effective treatment for cancer. Many therapeutic agents act by introducing sufficient DSBs into cancer cells to activate cell death pathways[25]. SDHB might affect human ovarian cancer cell chemosensitivity through DSB. In addition, colony formation assay was used to prove the result.

Conclusions

In current study, our research suggested *SDHB* played an important role in chemosensitivity. Through *SDHB* silencing, cancer cells could reduce the chemosensitivity to DDP, while *SDHB* over-expression

induced cancer cells more sensitive to chemotherapy. *SDHB* might be a target to overcome drug resistance in human ovarian carcinoma.

Declarations

Competing Interests: The authors have declared that no competing interests exist.

Acknowledgements

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Figures

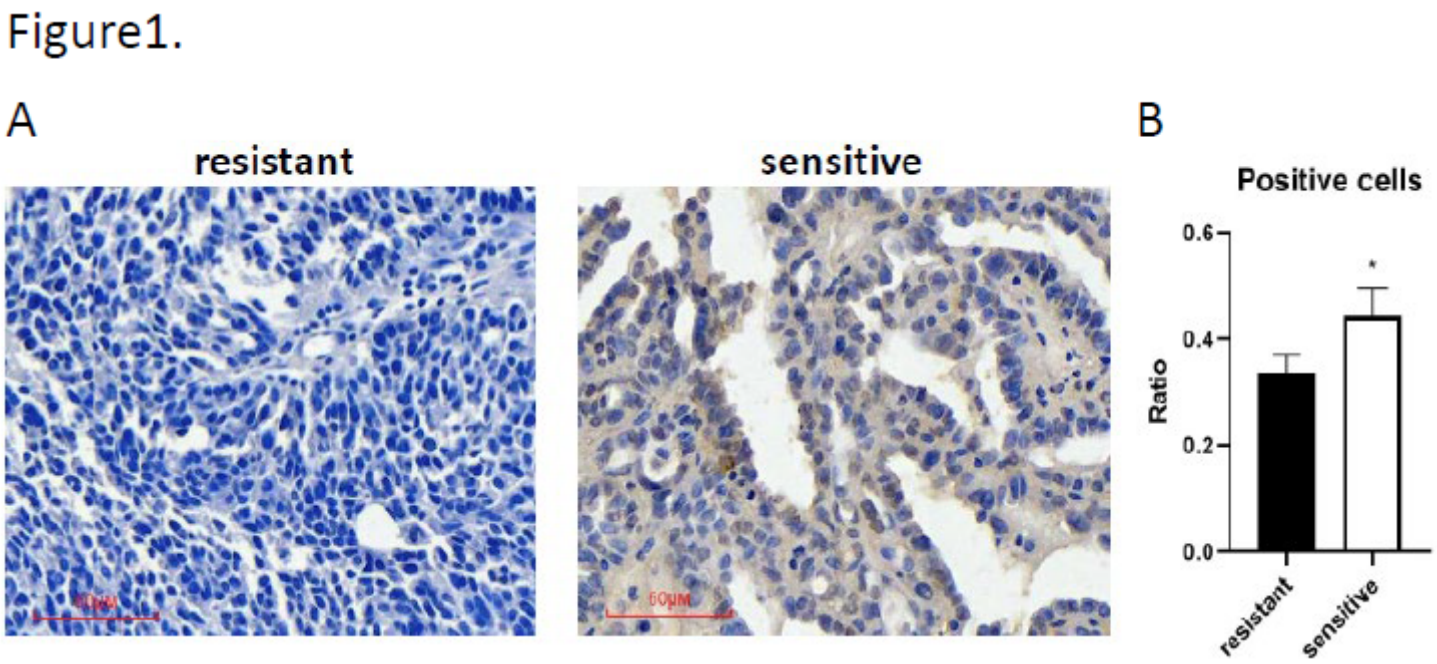

Figure 1

SDHB expressed lower in human ovarian cancer cisplatin-resistant tissues than the corresponding cisplatin-sensitive tissues. (A) Representative SDHB immunostained section is shown from the chemosensitive patient group and chemoresistive patient group. (B) The numbers of cases with the indicated level of SDHB expression in chemosensitive and chemoresistant group are shown.

Figure2.

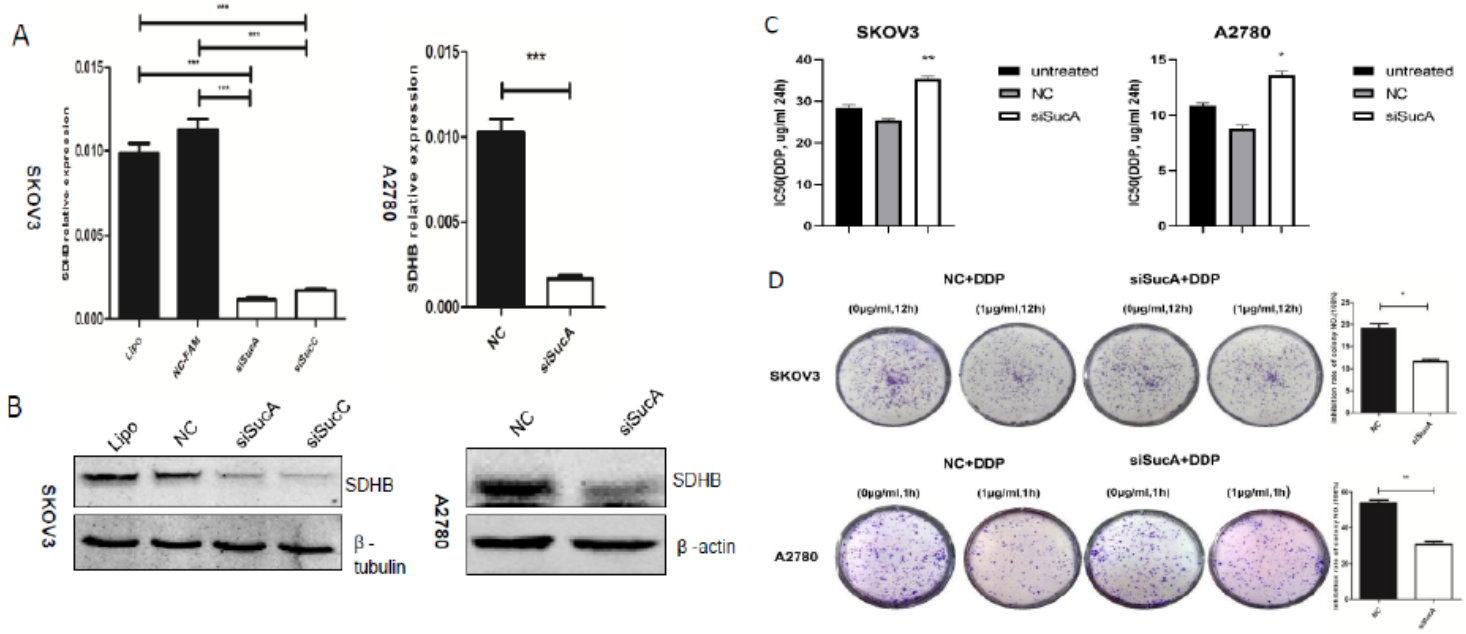

Figure 2

SDHB silencing increased drug resistance of ovarian cancer cells to DDP. (**A, B**) SKOV3 and A2780 cells were treated with siSucA oligonucleotides for 24 h and 48 h, then real-time PCR and Western blot was performed to detect *SDHB* mRNA and protein levels (***P< 0.001). **(C)** SKOV3 and A2780 cells were treated with *SDHB* siRNA oligonucleotides for 48 h. Then cells were treated with different concentrations of DDP for 24 h, cell viability was measured after incubation with CCK8 for 1.5 h (**P<0.01, *P<0.05). (**D**) Colony formation assay of *SDHB*-silenced SKOV3 and A2780 cells. Cells were cultured in 6-well plates for 8 d. The colonies were stained with crystal violet and scored (**P<0.01□*P<0.05).

Figure3.

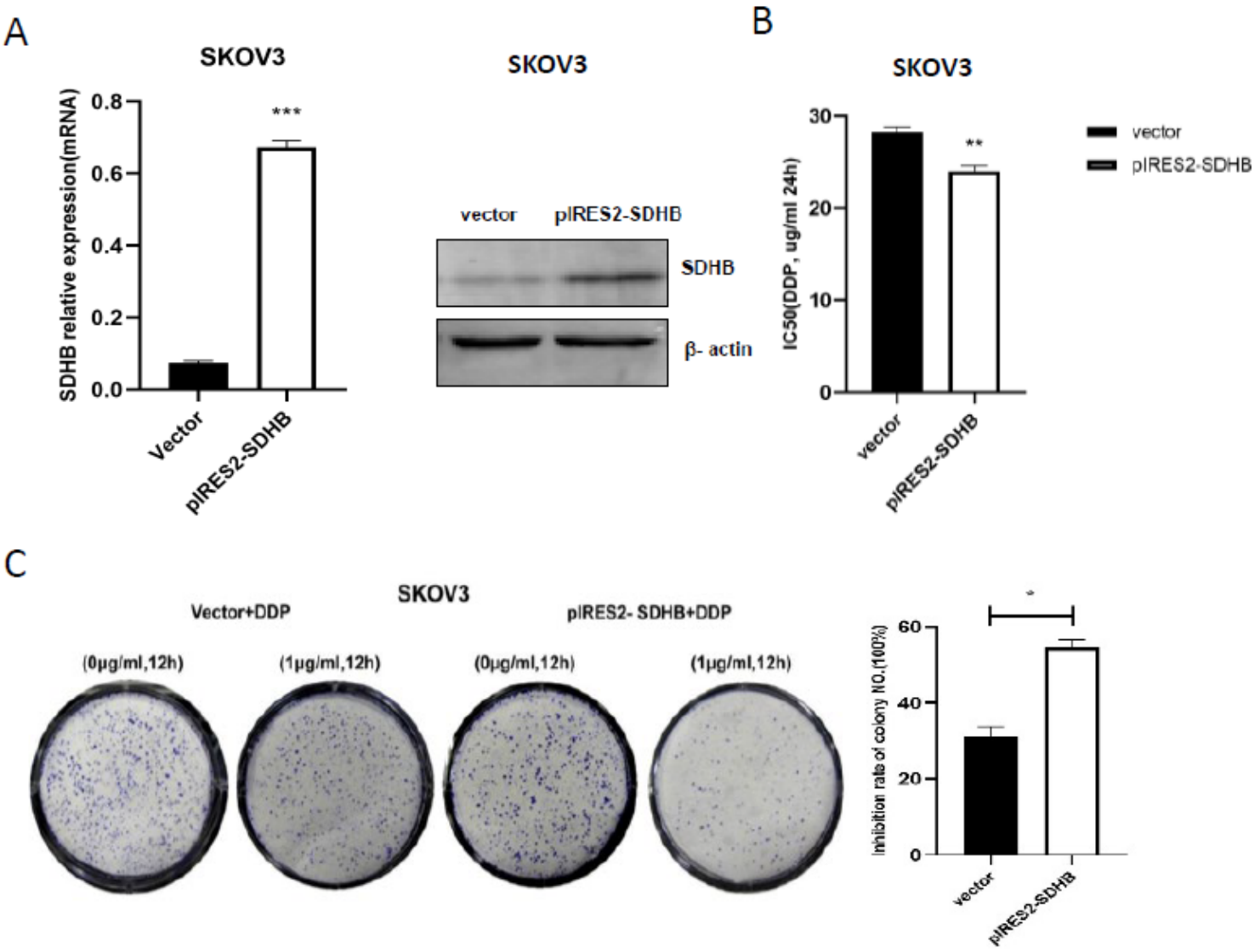

Figure 3

SDHB overexpression inhibited chemosensitivity of ovarian cancer cells to DDP. (**A**) SKOV3 cells were treated with pIRES2–EGFP vector (pIRES2-SDHB) or empty vector for 24 h and 48 h, then *SDHB* mRNA and protein levels were analyzed by Western blot (***P< 0.001). (**B**) SKOV3 cells were treated with *SDHB* overexpression vector for 48 h. Then cells were treated with different concentrations of DDP for 24 h, cell viability was measured after incubation with CCK8 for 1.5 h (**P<0.01). (**C**) Colony formation assay of *SDHB*-overexpressed SKOV3 cells. Cells were cultured in 6-well plates for 8 d. The colonies were stained with crystal violet and scored (*P<0.05).

Figure4.

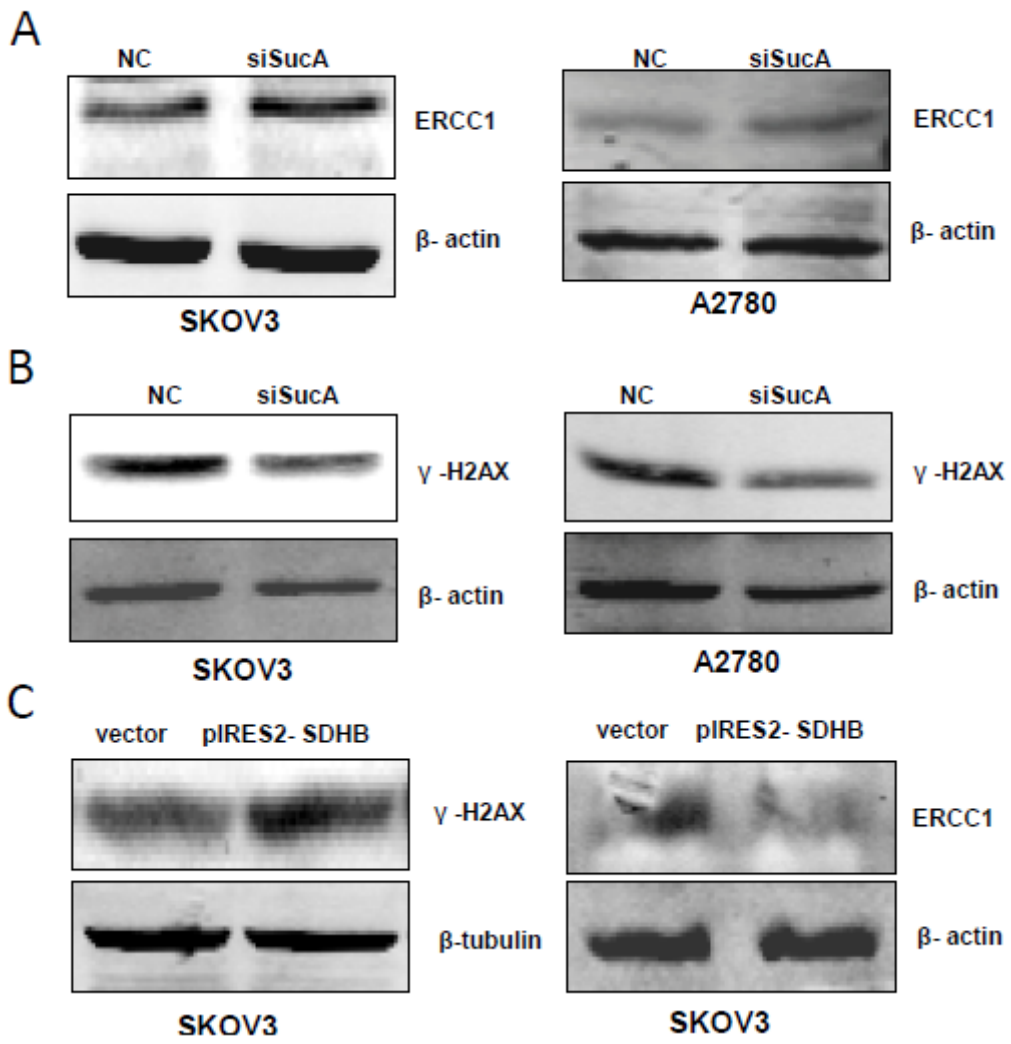

Figure 4

SDHB expression affected the DNA repair damage related proteins in SKOV3 and A2780 cells. (**A**&**B**) After transient transfection with *SDHB* siRNA for 48 h in SKOV3 and A2780 cells, ERCC1 and γ-H2AX protein levels were compared between the NC and siSucA group with b-tubulin or b-actin used as a loading control. (**C**) SKOV3 cells were treated with pIRES2–EGFP vector (pIRES2-SDHB) or empty vector for 48 h, ERCC1 and γ-H2AX protein levels were analyzed in *SDHB*-overexpressed SKOV3 cells.