Targeting ACLY Attenuates Tumor Growth and Acquired Cisplatin Resistance in Ovarian Cancer by Inhibiting PI3K/AKT Pathway and Activating AMPK/ROS Pathway

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Research

Keywords: ACLY, ovarian cancer, cisplatin resistance, PI3K/AKT pathway, AMPK/ROS pathway

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Abstract

Background: Ovarian cancer is the most lethal female genital malignancy. Though cisplatin is still the first-line chemotherapy to treat ovarian cancer patients with debulking surgeries, its efficacy is limited due to the high-incidence of cisplatin resistance. ATP citrate lyase (ACLY) has been proved to be a key metabolic enzyme and was related to poor prognosis in various cancer, including ovarian cancer. Nevertheless, there has not been any research elucidating the relationship between ACLY and cisplatin resistance and the mechanism of how it works.

Methods: Survival analysis was mainly carried out on the website. Bioinformatic analysis was performed in R/R studio. Proliferative activity was measured by MTT assay and colony formation assay. Cell cycle and apoptosis analysis were performed by flow cytometry. Acquired cisplatin resistant cell line A2780/CDDP was generated from A2780 by exposing to gradually elevated concentration of cisplatin. MTT assay was used to calculate IC50 of cisplatin. Xenograft tumor assay was used test cell proliferation in vivo.

Results: Higher expression of ACLY was found in ovarian cancer tissue and related to poor prognosis. Knockdown of ACLY in A2780, SKOV3 and HEY cells inhibited cell proliferation, caused cell cycle arrest by modulating P16/CDK4/CCDN1 pathway and induced apoptosis probably by inhibiting p-AKT activity. Bioinformatic analysis of GSE15709 dataset revealed upregulation of ACLY and activation of PI3K/AKT pathway in acquired cisplatin resistant cells, in line with the results of A2780/CDDP cells generated by us. Knockdown of ACLY could alleviate cisplatin resistance and work synergistically with cisplatin treatment in inducing apoptosis in A2780/CDDP cells, by inhibiting PI3K/AKT pathway and activating AMPK/ROS pathway. ACLY specific inhibitor SB-204990 also showed the same effect. In A2780/CDDP cells, AKT overexpression could destroy cisplatin re-sensitization caused by ACLY knockdown.

Conclusions: Knockdown of ACLY attenuated cisplatin resistance by inhibiting PI3K/AKT pathway and activating AMPK/ROS pathway. These findings suggested that combination of ACLY inhibition and cisplatin could be an effective strategy for overcoming cisplatin resistance in ovarian cancer.

Introduction

Ovarian cancer is one of the most lethal tumors in the world. Asymptomatically, in most cases, tumors have already spread to other pelvic or extra-pelvic organs when diagnosed. To date, in all female malignant tumors, ovarian cancer accounted for 5% of all deaths in patients with an incidence rate of only 2.5%[1]. The standard therapy is debulking surgery with platinum-based chemotherapy [2]. Although 60%-90% of patients with ovarian cancer response well to first-line platinum-based chemotherapy[3], median PFS of advanced ovarian cancer patients is about 18 months, as most recurrent patients are platinum-sensitive. Typically, for platinum-sensitive patients, platinum-based regiments continued as chemotherapy strategy until the patients developed to resistant ones. [4]. Many reasons accounted for acquired platinum resistance, including the reduced accumulation of the drug [5], increased levels of
glutathione[6] and metallothionein[7], and enhanced DNA repair[8, 9]. Therefore, it is urgent to find ways to prevent acquired platinum resistance to maintain the well response to platinum.

ATP citrate lyase (ACLY) is a tetramer consisting of four identical subunits, activated by phosphorylation on catalytic phosphorylated His760 residue of each N-terminal subunit[10], locating in cytoplasm, mitochondria and nucleus[11]. ACLY catalyzes the synthesis of citrate and coenzyme A (CoA) into oxaloacetate (OAA) and acetyl-CoA [9]. Acetyl-CoA participates in de novo synthesis and catalyzes acetylation of proteins, especially in acetylation of histones and transcription of certain proteins. OAA is a substrate to produce aspartate, which is required for nucleotide and polyamine synthesis, and also sustains the regeneration of NAPDH/H+. NADH/H+ participates in redox reaction and biosynthesis[12]. ACLY connects glucose metabolism with de novo lipid synthesis and acts as a key enzyme in de novo lipid synthesis. High expression of ACLY were detected in many kinds of tumors, including non-small cell lung cancer, colorectal cancer, renal cancer, epithelial ovarian cancer, prostate cancer, breast cancer, bladder cancer, hepatocellular cancer, and glioblastomas[13]. Targeting ACLY in tumor therapy seems to be a novel strategy.

Our team has previously explored ACLY as a prognostic factor of ovarian cancer, also demonstrated that inhibition of ACLY suppressed the proliferation of ovarian cancer cells[14]. With bioinformatic analysis on acquired cisplatin-resistant ovarian cancer cells versus cisplatin-sensitive ones, we found that ACLY and its related pathway were significantly upregulated in cisplatin-resistant cells.

Through bioinformatic analysis of GEO dataset, we first recognized ACLY to be a key enzyme in regulating acquired platinum resistance. Through investigating the re-sensitization effect of ACLY knockdown on acquired platinum resistant cells, our findings proposed ACLY as a novel target for maintaining sensitization of ovarian cancer to platinum.

Materials And Methods

Bioinformatic analysis

The survival analysis of TCGA datasets and GEO datasets in ovarian cancer patients were performed on website (http://kmplot.com/analysis/index). GEO datasets (GSE15709) were used, data were downloaded from website (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15709), and analyzed by R/R studio. DEGs (differential expressing genes) were extracted by limma R package, with the filter standard of log|FC| ≥ 1 and p value<0.05. GO and KEGG pathway enrichments were performed by clusterProfiler R package[15]. The visualization of pathway was performed by Pathview R/Bioconductor package[16]. We calculated rich factor of KEGG enrichment comprehensively with gene numbers and weights using S4Vectors R package.

Cell lines and culture conditions
The human epithelial ovarian cancer cell lines A2780, SKOV3 and HEY were purchased from the Cell Bank of the Chinese Academy of Sciences in Shanghai, China. The HEY cells were cultured in DMEM with 10% fetal bovine serum, A2780 and SKOV3 cells were cultured in RPMI-1640 medium along with 10% fetal bovine serum. All kinds of the culture medium were added 100U/ml penicillin and 100μg/ml streptomycin. Cells were maintained at 37°C with 5% CO₂ and 95% air.

**Induction of cisplatin resistance**

The parental A2780 cells were exposed to repeated and gradual elevated concentration of cisplatin (MCE, NJ, USA) over a year period, with the concentration of cisplatin gradually increasing from 2μM to 50μM.

**Cell viability and colony formation assays**

MTT assay was used to detect cell proliferative ability. To examine the proliferative ability, cells were inoculated into 96-well plates, each well contained 800-1000 cells, incubated overnight to adhere. At fixed time from day 1 to day 5, 10μl MTT solution (5mg/ml, sigma, CA, USA) was added into every well, then put the plate back to incubate at 37°C for 4H. The supernatant was carefully suctioned and 100μl DMSO (MP Biomedicals, OH, USA) was added into every well. After formazan fully dissolved, put the plate in the microplate reader (Tecan Group Ltd, Männedorf, Switzerland), reading the absorbance at 490nm.

For colony formation assays, 800 single cells were seeded in 6-well plate and mix with 2ml cultural medium. After incubated at 37°C for 12 days, cells were fixed with methanol and stained with 0.1% crystal violet (Beyotime, Beijing, China).

**Drug resistance assay**

MTT assay was used to measure the surviving fractions and IC50 of cells. Cells were seeded into 96-well plate with cell concentration of 3000 cells per well, after exposure to different concentration of cisplatin and incubating for 24-72H. Finally, results were measured by microplate reader as mentioned above.

**Cell cycle assay**

Cells were harvested and fixed with ethanol when density of cells reached 80%, then stained with propodeum iodide according to protocol (BD, NJ, USA). The treated cells were harvested by flow cytometer (FACSCalibur, BD, USA). The results analyzed through Modifit LT software.

**Cell apoptosis assay**

Cells transfected with lentivirus or exposure to different concentration of cisplatin were harvested with tryrisin without EDTA, washed with PBS for 3 times, resuspended with Annexin binding buffer reaching a concentration of $1 \times 10^6$ per 100μl. Then cells were dyed with 5μl Annexin V-APC and 10μl 7-AAD (MultiSciences, Hangzhou, China), incubated with room temperature for 5 minutes. Finally, cells were collected with flow cytometer (FACSCalibur, BD, USA), and analyzed with Flowjo V10 software.
**Lentivirus production and stable transfections**

The plasmid containing shACLY and corresponding negative control (NC) were purchased from Genechem (Shanghai, China). The HEK279T cells were used to produce lentivirus. The shACLY plasmid were transfected in HEK293T cells together with pMD2.G and psPAX2. The obtained lentivirus was used to infect ovarian cancer cells for 12H. Cells were cultured with medium containing 2 μg/mL puromycin (Solabio, Beijing, China) to get the stable transfected cells.

**Transient transfection**

The plasmid containing AKT and corresponding NC were purchased from Genechem (Shanghai, China). Lipo3000 (Invitrogen, USA) was used to transfected plasmid into cells. Cells then harvested after 24–48 h for the following assays

**Western blotting assays**

Adherent cells were washed by PBS for 3 times, lysed with RIPA Lysis Buffer (Beyotin, Beijing, China) with 1% PMSF and 1% NaF, placed on the ice for 30 minutes. The contents were collected and centrifuged to get the supernatant as protein. The concentration of protein was measured by BCA reagents assays (Beyotime, Beijing, China). Adequate volume of 5×loading buffer was added into the protein and reaching the final concentration of 1×, and followed by metal bath. Samples were loaded into SDS-PAGE (10% separation gel or 12% separation gel) with 30μg per well, electrophorese to separate the protein in the page, then transferred to 0.22-μm polyvinylidene fluoride membranes (Merck Millipore, USA). Membranes were blocked in 5% skimmed milk for 1 hour, then incubated in primary antibodies overnight in 4℃, washed with 1×TBST, incubated in HRP-linked secondary antibodies respectively at room temperature for 1.5H or less. The chemiluminescent substrate (Thermo Fisher Scientific Inc, MA, USA) were used to detect bands on membranes under Image Quant LAS 4000 (GE Healthcare Life Science). GAPDH was detected as the endogenous control.

**Tumor xenograft experiment**

The A2780 and A2780/CDDP cells stably expressing shACLY and corresponding NC cell lines were used to construct in this procedure. Cells were harvested, counted to 1×10^7 and resuspended in 200μl PBS were injected subcutaneous on either side of axilla of the 4-6week old nude female mice. Tumor sizes were measured every other day from day 10 after injection. The mice compromise A2780/CDDP cells were received cisplatin injection intraperitoneally with a concentration of 4 mg/kg body weight (B.W.) on day 7, 14 and 28 (total three injections). Mice were sacrificed 35 days after A2780 cells injection and 4 weeks after cisplatin injection in A2780/CDDP cells group. The volumes of tumors were calculated as V= [(length × width^2)/2].

**Antibodies and chemical inhibitors**
The antibodies of ACLY, p16 ARC and PFKM were purchased from Abcam (Cambridge, UK). The antibodies of cleaved PARP, TP53, pan-AKT, phosphorylated-AKT (Ser473), phosphorylated-AMPK-α and GAPDH were purchased from Cell Signaling Technology (MA, USA). SB-204990 was purchased from MCE (NJ, USA).

**Measurement of intracellular ROS levels**

The intracellular ROS levels were measured using a Reactive Oxygen Species Assay Kit (Beyotime Shanghai, China). 3000 cells were seeded in each well of 96-well plates as and exposed to 20μM of cisplatin for 24H. Following the treatment, the cells were incubated with DCFH-DA for 20min at 37°C and then measured at 488 nm excitation and 525 nm emission by a fluorescence microplate.

**Statistical analysis**

All experiments were repeated at least 3 times. GraphPad Prism 8.0.1 (GraphPad Software, USA) was mainly used in data analyzing. Student's t test and one-way ANOVA analysis were applied to determine the statistical differences among different groups. Persistent variable was presented mainly by means±SD. IC50 of cells were presented as best fit value with 95%CI. P <0.05 was regarded as statistically significant.

**Results**

1. **ACLY was upregulated in ovarian cancer tissues and was associated with poor prognosis.**

   Firstly, we performed bioinformatic analysis to explore the characteristics of ACLY in ovarian cancer. We explored mRNA level of tissues from Qilu hospital (T = 23, N = 23), and found that ACLY was upregulated in cancer tissues compared with normal ones, with statistical significance \( P= 0.0206 \) (Fig. 1A). To elucidate the relation between ACLY expression and prognosis (mainly measured by overall survival), TCGA datasets and GEO datasets were used to perform bioinformatic analysis. In the five datasets we analyzed, higher expression of ACLY predicted poorer overall survival results (Fig. 1B-1F).

2. **ACLY knockdown inhibited ovarian cancer progression in vitro and in vivo.**

   A2780, SKOV3 and HEY was selected for following experiments from 6 common ovarian cancer cell lines as they expressed relatively higher ACLY (Fig. 2A). Lentivirus carried shACLY and NC was transfected into and stably expressed in cells, with western blotting confirming the blocking effectiveness (Fig. 2B). MTT assay revealed that knockdown of ACLY can significantly suppress the proliferative ability of ovarian cancer cells (figure. 2C), in keeping with that less colonies formed in shACLY cells compared to NC cells (Fig. 2D). In cell cycle analysis, we found that G0/G1 phase took larger proportion in ACLY knockdown cells, which demonstrated that knockdown of ACLY induced cell cycle arrest of G1 phase (Fig. 2D). We found the expression of G1 phase checkpoint cyclinD1 (CCND1) and CDK4 was corporately
downregulated in ACLY knockdown cells. P16, as the upstream inhibitor of CDK4, upregulated upon ACLY knockdown cells (Fig. 2G). The apoptosis assay results showed knockdown of ACLY caused an increase apoptosis (Fig. 2F). While compared with P53 mutant cell line (SKOV3), P53 wild type cell line (A2780 and HEY) gained higher proportion of apoptotic cells and P53 upregulation upon ACLY knockdown (Fig. 2G). We detected that cleaved parp was significantly upregulated in ACLY knockdown cells (Fig. 2G). To further verify the anti-tumor effect, we used A2780 cell with lentivirus stably transfecting shACLY and NC to construct in vivo model. The volume of tumors was significantly smaller in ACLY knockdown group than those in NC group, and same trend was observed in tumor weight respectively (Fig. 2H).

3. ACLY was upregulated in A2780/CDDP cells through bioinformatic analysis.

We screened DEGs between A2780 versus A2780/CDDP with GEO datasets GSE15709. We found ACLY was upregulated in A2780/CDDP cells (Fig. 3A). The volcano plot of DEGs was presented (Fig. 3B). We then performed enrichment of DEGs via GO pathways and KEGG pathways (Fig. 3C). We observed obvious activation of PI3K/AKT pathway in A2780/CDDP cells (Fig. 3C-E). Additionally, we found that AMPK was downregulated in A2780/CDDP cells (Fig. 3E). With the result that knockdown of ACLY downregulated p-AKT in ovarian cancer cells (Fig. 2G), We proposed that ACLY knockdown might rescue the cisplatin resistance caused by inhibiting PI3K/AKT pathway.

4. ACLY knockdown re-sensitized A2780/CDDP cell to platinum

Firstly, MTT assays and colony formation assays were used to confirm the cisplatin resistance of A2780/CDDP cells, IC50 of A2780/CDDP cells was nearly five times as that of A2780 cells of 48H results, and more than 10 times of 72H results (Fig. 4A). Colony formation assays also indicated that A2780/CDDP showed more resistant to cisplatin (Fig. 4B). MTT assays showed that ACLY knockdown in A2780/CDDP cells inhibited proliferative, colony formation and IC50 of cisplatin (Fig. 4C, D and E). Colony formation assay revealed that the survival ratio of cisplatin treatment cells was higher in A2780/CDDP-NC cells compared with A2780/CDDP-shACLY cells (Fig. 4F). Apoptotic proportion of A2780/CDDP-shACLY cells was significantly higher than that of A2780/CDDP-NC cells, and difference increased with time and concentration (Fig. 4G).

5. ACLY knockdown inhibited PI3K/AKT pathway and activated AMPK pathway.

The results of western blotting detected that PI3K, pan-AKT, p-AKT were upregulated, and p-AMPK-α were downregulated in A2780/CDDP cells (Fig. 5A), which in accordance with bioinformatic analysis of pathway enrichment of GSE15709. Then we measured the change of these factors in cells treated with ACLY knockdown allied cisplatin. As shown in Fig. 5B, p-AKT was downregulated under cisplatin treatment and combination with ACLY knockdown drove the downregulation. PI3K was downregulated under treatment of cisplatin, but encountered sharp downregulation when ACLY knockdown joined. ACLY
knockdown synergistically activated AMPK-α with cisplatin treatment. Accordance with the activation of AMPK-α, reactive oxygen species (ROS) increased, especially when combined ACLY inhibition and cisplatin treatment (Fig. 5C). To verify the synergistic anti-tumor effects of ACLY inhibition and cisplatin in vivo, we used A2780/CDDP-shACLY and A2780/CDDP-NC cells to construct tumor models in nude mice. Tumor volumes of A2780/CDDP-shACLY cells were smaller than those of A2780/CDDP-shNC cells, and tumor weight was lighter (Fig. 5D).

SB-204990 is a highly effective and specific small molecule inhibitor of ACLY. With the low dose of SB-204990 (less than 30 µM), we saw no significant difference in proliferation of treated cells (Fig. 5E). While, when combined with cisplatin, it showed synergistic effect with cisplatin in reducing IC50 of cisplatin over time (Fig. 5F).

6. Overexpression of AKT increased cell resistance to cisplatin in A2780/CDDP-shACLY cells.

We supposed that knockdown of ACLY reduced resistance to cisplatin via downregulating PI3K/AKT pathway. Then we used AKT overexpression to do the rescue experiment, Western blotting verified the overexpression of AKT (Fig. 6A). We measured whether overexpression of AKT affected cisplatin sensitivity in A2780/CDDP-shACLY cells and found a recovery of IC50 in A2780/CDDP-shACLY-AKT cells compared with A2780/CDDP-shACLY-NC cells (Fig. 6B). We then wondered whether the overexpression could rescue the higher apoptosis induced by knockdown of ACLY combined with cisplatin treatment. Flow cytometry result showed overexpression of AKT could partly rescue the apoptosis induced by ACLY inhibition combined cisplatin treatment (Fig. 5C). Cleaved parp detected by western blotting also showed the same trend in AKT overexpression cells (Fig. 5D).

Discussion

Ovarian cancer appears to be the most lethal disease in female. The two main reasons accounting for this are asymptomatic at early stage and platinum resistance (intrinsic or acquired). Although the initial treatment gained remission, recurrence is always ensued with acquired resistance to platinum and further chemotherapy[17]. It’s urgent to find ways to overcome resistance to platinum.

Proliferation at a rapid pace is characterized in cancer tissues and cancer immortalized cells, resulting in the extremely active glucose metabolism and lipid metabolism. Proliferation requires a large amount of cell membrane synthesis and fatty acid synthesis[18]. In tumor tissues, enhancement of de novo lipid synthesis has been detected[19]. In bladder cancer and hepatocellular carcinoma, several lipogenic enzymes were detected higher expression in tumor tissue than normal tissues, which associated with poor prognosis [20, 21]. Qian et al. revealed that high ACLY expression correlated with poor prognosis, advanced stages and lymph node metastasis in gastric cancer[22]. Toshiro Migita et al. elucidated that ACLY gained higher expression in non-small cell lung cancers than normal lung tissues, correlating with advanced stage, grade, and poorer prognosis[23]. Similar effect of ACLY was observed in renal cell
carcinoma in the work of Teng et al. issued on 2013[24]. Toshiro Migita et al. revealed that ACLY depletion induced growth suppression and apoptosis in a subset of cancer cell lines including prostate cancer, breast cancer, colorectal cancer cell lines[25]. Jinghan Wang et al. published a research that low ACLY expression was associated with better overall survival in AML patients, and knockdown ACLY in THP-1 and MOLM-13 leukemia cell lines caused proliferation arrest[26].

Our previous study has revealed higher mRNA expression of ACLY in ovarian cancer tissue compared with normal ovarian epithelium, and the higher expression predicted poorer prognosis with shorter overall survival[14]. Furthermore, we explored GEO datasets and TCGA datasets, and got the similar results.

Most research focused on the influence factor modulating ACLY. Sterol Regulatory Element-binding Protein (SREBP-1) transcriptionally regulated ACLY expression[27]. Akt directly activates ACLY by phosphorylation of ACLY[23]. Acetylation at lysine residues of ACLY stabilized ACLY by competitive inhibited ubiquitin degradation[28].

Only a few researches explored the mechanisms of ACLY knockdown in malignant tumors. JUN-ICHI HANAI et al. revealed that knockdown of ACLY attenuated PI3K/AKT pathways, and combination with statin would receive dual inhibition of lung cancer growth[29]. Toshiro Migita et al. has proved that ACLY inhibition may induce ROS via AMPK pathway[30].

Even fewer researches paid attention to connection of ACLY knockdown and chemo-therapy resistance. ACLY knockdown could re-sensitize SN38 resistant colorectal cancer cells[31]. Targeting ACLY sensitizes castration-resistant prostate cancer cells to AR antagonism by ACLY-AMPK-AR feedback pathway[32]. In pleural mesothelioma cells, Zhang XD et al. found that exogenous addition of citrate could induce apoptosis and act as synergistic effect with cisplatin[33].

In our current work, we found ACLY knockdown inhibited cell proliferation and induced apoptosis in three cell lines of ovarian cancer. In ACLY knockdown cells, cell cycle arrested in G1 phase, with a decreased expression of proteins related to G1 phase cell cycle check point, including CCND1, CDK4. Their upstream regulator, P16 was examined to be upregulated in ACLY knockdown cells. P16 was a canonical negative regulator of CDK4[34]. Endogenous depletion of ACLY has been found to be related to decreased p-AKT expression in lung cancer cells[29], in consistence with our results. To further verify the anticancer property of ACLY inhibition, we performed tumor formation assays in nude mice and observed same trend as in vitro experiment. To date, our study elucidated that knockdown of ACLY inhibited cell proliferation in vitro and in vivo, probably via modulating P16-CCND1-CDK4 pathway and inhibiting p-AKT activity.

Additionally, we observed an increase of the expression of P53 in P53 wild-type cells as A2780 and HEY, but not in P53 mutant cells as SKOV3. As is well-known, P53 plays a vital role in anti-tumor effects and P53 activation inhibits cell cycle as well as increases apoptosis[35]. Coincidently, we found that the increase of apoptosis in SKOV3 was less obvious than that of A2780 and HEY on the knockdown of ACLY. Maybe the difference of P53 status may explain that.
AKT, or protein kinase B is a canonical downstream effector of PI3K[36, 37]. The PI3K/Akt pathway has been well studied as a molecular ladder for cells to avoid death[38]. And in chemotherapy resistance, it has been well known that activation of PI3K/AKT pathway not only appeared but also played as an important role in multi-drug resistance[39]. According to Yang XK et al., AKT promoted chemoresistance to cisplatin in ovarian cancer cell lines, by modulating P53 on the caspase-dependent mitochondrial death pathway[40]. Amy S et al. have reported that AKT activation promoted breast cancer cell survival and therapeutic resistance, and induction of AKT by trastuzumab or tamoxifen treatment reduced the apoptosis induced by doxorubicin[41]. In breast cancer, Knuefermann C reported that HER2/PI3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells[42]. In addition, Perez-Tenorio G reported activation of AKT/PKB related to resistance to endocrine therapy[43].

In our current study, by bioinformatic analysis, we found correlations between high ACLY expression and acquired cisplatin resistant ovarian cancer cells. After performing pathway enrichment of differential expressed genes, we noticed that upregulated of PI3K/AKT pathway in resistant cells was distinct. We generated A2780/CDDP (A2780 acquired cisplatin resistant cell line). we used Western blotting to examine expression of proteins related to PI3K/AKT pathway, found that PI3K and AKT, including p-AKT were upregulated in A2780/CDDP cell line, as the same trend with bioinformatic analysis of GSE15709.

Since AKT played a positive role in cancer chemotherapy resistance, and inhibition of ACLY downregulated PI3K/AKT pathway, we then wondered whether knockdown of ACLY can re-sensitize A2780/CDDP cells to cisplatin. We at first hypothesized that knockdown of ACLY may decrease IC50 of cisplatin in A2780/CDDP cells, which was then proved by MTT results. We performed cell apoptosis assays on A2780/CDDP-NC and A2780/CDDP-shACLY cells in dose-dependent and time-dependent manner, and found that A2780/CDDP-shACLY responded more sensitive to cisplatin. We also performed in vivo tumor formation assays, ACLY knockdown combining with cisplatin treatment received much better curative effect. By far, we verified that the knockdown of ACLY could reduce cisplatin resistance in ovarian cancer in vitro and in vivo.

Sb-204990 is a highly effective and specific small molecule inhibitor of ACLY. Studies have shown that SB-204990 can effectively reduce cholesterol and fatty acid synthesis of hepatocytes at a concentration of 30uM, without affecting the proliferation of normal hepatocytes[44]. In experimental animal models, SB-204990 was verified to reduce serum cholesterol and fatty acid content, but did not affect the survival time and other physiological indicators of animals. At present, this inhibitor has been applied in clinical practice. In diabetic patients, SB-204990 can effectively inhibit platelet aggregation, reduce the occurrence of complications, and improve the prognosis of diabetic patients[45]. The research of its effects on tumor is still limited to cell and animal experiments, but it has shown good anti-tumor effects in various tumor cells and tumor forming models[46]. In our study, we verified that low-dose SB-204990 did not affect the proliferative activity of A2780/CDDP cells by MTT assays, while when co-interacted with cisplatin, it could alleviate the resistance to cisplatin by reducing the IC50 of cisplatin, which meant they worked in synergistic way. SB-204990’s successful use in diabetic patients make it a possible regiment for patients with ovarian cancer in chemotherapy combined with platinum.
With the knockdown of ACLY in A2780/CDDP cells, the expression PI3K, AKT, p-AKT went down, thus we hypothesized that ACLY worked through PI3K/AKT pathways. Then we performed rescue experiment with AKT overexpression and verified our hypothesis.

AMPK pathway plays a critical role in metabolic reprogramming, which is also critical in multidrug resistance in cancer therapy. The famous anti-diabetic drug, Metformin, also as a well-known AMPK activator, can increase cancer chemosensitivity in vitro and in vivo[47]. Toshiro Migita et al. has proved that ACLY inhibition may induce ROS via AMPK pathway[30]. In our study, knockdown of ACLY activated AMPK by an increase of p-AMPK-α, at the same time, ROS was elevated in ACLY knockdown A2780/CDDP cells. As we mentioned before, the toxic effect of cisplatin was to produce excessive ROS to induce apoptosis, the higher ROS produced by the combination of ACLY inhibition and cisplatin treatment, may, explain the synergetic effect.

Limitation of the study was that we did not include primary cisplatin resistance in the study. Due to heterogeneity of ovarian cancer patients, it's better we do patient derived tumor Xenograft or patient derived cells to reflect the status of primary cisplatin and remain the heterogeneity.

Conclusions

We explored ACLY was upregulated in ovarian cancer tissue and correlated with poor prognosis. Knockdown of ACLY caused cell cycle arrest by activating P16 thus inhibiting its downstream CDK4/CCND1, caused apoptosis probably by inhibiting p-AKT. We found that knockdown of ACLY reduced resistance to cisplatin in acquired cisplatin resistant cells, by inhibiting PI3K/AKT pathway and activating AMPK/ROS pathway. We found the novel function of SB-204990, the specific inhibitor of ACLY, might be of use in clinical for ovarian cancer patients. While, there need to be more researches on the heterogeneity of patients derived tumors. Here we in this article, revealed a novel character of ACLY played in acquired cisplatin resistance ovarian cancer cells.

Abbreviations

ACLY: ATP citrate lyase; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT: Protein kinase B; IC50: 50% inhibiting concentration; CDK4: Cyclin Dependent Kinase 4; CCND1: Cyclin D1; AMPK: AMP-activated protein kinase; ROS: Reactive oxygen species; PFS: Progression-free survival; DEGs: Differential expression genes; TCGA: The cancer genome atlas program; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes.

Declarations

Ethics approval and consent to participate

The animal experiment was approved by the Experimental Animal Ethics Committee of Qilu Hospital of Shandong University (Approval number: DWLL-2019-011). The studies involving human participants were
reviewed and approved by Ethics Committee of Shandong University (Approval number: (KYLL-2019-261).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The survival analysis of TCGA datasets and GEO datasets in ovarian cancer patients were performed on website (http://kmplot.com/analysis/index). GEO datasets (GSE15709) were used, data were downloaded from website (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15709).

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

XW, YW and PSL designed the research process. XW, XW and WL performed the bioinformatic analysis. JJS and YXP performed the experiments. XXM and QHL analyzed the data. XW, YXP and RL wrote the paper. All authors read and approved the final manuscript.

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Figures

A

B

TCGA
ovarian cancer

C

GSE
15622

D

GSE
18520

E

GSE
23554

F

GSE
30161

Figure 1
ACLY was upregulated in ovarian cancer tissues, and its expression was associated with poor prognosis. (A) The comparison of ACLY expression between cancer tissue and normal tissue of patients from Qilu hospital. (B-F) Overall survival analysis based on ACLY expression (high-expression group vs. low-expression group) of TCGA ovarian cancer cohort, GSE15622 dataset, GSE18520 dataset, GSE23554 dataset, GSE30161 dataset.

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ACLY knockdown inhibited ovarian cancer progression in vitro and in vivo. (A) Western blotting was used to detect the expression of ACLY in several ovarian cancer cell lines. (B) Western blotting was used to confirm the effectiveness of ACLY knockdown by lentivirus in A2780, SKOV3 and HEY cells. (C) MTT assays were used to detect the proliferative activity change on the knockdown of ACLY in A2780, SKOV3 and HEY cells. (D) Colony formation assays of ACLY knockdown and its corresponding NC cells of
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Figure 4

ACLY knockdown re-sensitized A2780/CDDP cell to platinum (A) 24h, 48h and 72h IC50 were measured after treatment of cisplatin with concentration gradients. 24H IC50 of A2780 and A2780/CDDP were 9.827(9.316-10.36)μM and 30.75(28.91-33.03)μM respectively, 48H IC50 were 4.593(4.355-4.838)μM and
22.68(20.88-24.89) μM respectively, 72H IC50 were 1.861(1.749-1.976) μM and 20.76(18.39-23.85) μM respectively. (B) Colonies formation assays and the survival ratio of A2780 and A2780/CDDP cells treated with different concentration of cisplatin. (C) MTT assays were used to measure the proliferative ability of ACLY knockdown A2780/CDDP cells and their corresponding NC cells. (D) Colony formation assays were performed and analyzed in ACLY knockdown cells and their corresponding NC cells. (E). IC50 of cisplatin of 24h, 48h and 72h after cisplatin gradients added. 24h IC50 in A2780/CDDP-NC cells and A2780/CDDP-shACLY cells were 31.71(26.49-38.15) μM and 16.64(14.26-19.30) μM respectively, 48h IC50 were 26.07(22.83-29.74) μM and 11.55(10.43-12.76) respectively, 72h IC50 were 14.16(12.31-16.19) μM and 4.648(4.138-5.227) respectively. (F) Colony formation assays were performed in the aforementioned cells under treatment with different concentration of cisplatin. (G) Apoptosis assays were measured in cells treated with different concentrations and different time of A2780/CDDP-NC and A2780/CDDP-shACLY cells. (H) Cleaved parp was measured and quantified in A2780/CDDP-NC and A2780/CDDP-shACLY cells with or without cisplatin treated, the concentration of cisplatin was 20 μM.
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ACLY knockdown re-sensitized A2780/CDDP cell to platinum (A) 24h, 48h and 72h IC50 were measured after treatment of cisplatin with concentration gradients. 24H IC50 of A2780 and A2780/CDDP were 9.827(9.316-10.36)μM and 30.75(28.91-33.03)μM respectively, 48H IC50 were 4.593(4.355-4.838)μM and 22.68(20.88-24.89)μM respectively, 72H IC50 were 1.861(1.749-1.976)μM and 20.76(18.39-23.85)μM respectively. (B) Colonies formation assays and the survival ratio of A2780 and A2780/CDDP cells
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Figure 5

ACLY knockdown inhibited PI3K/AKT pathway and activated AMPK pathway. (A) Western blotting was used to detect the differential expression of ACLY, PI3K/AKT pathway and p-AMPK-α in A2780 and A2780/CDDP cells, the bands were quantified and analyzed. (B) Western blotting on A2780/CDDP-NC and A2780/CDDP-shACLY cells, and them under 20μM cisplatin treatment for 48H, the bands were quantified and analyzed. (C) ROS production of the aforementioned cells and them under treatment of
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**Figure 6**

A

| A2780/CDDP | shACLY | AKT | pan-AKT |
|------------|--------|-----|---------|
| -          | -      | -   | +       |
| +          | -      | +   | +       |
| -          | +      | +   | +       |

B

![Graphs showing cell viability at 24h, 48h, and 72h for different treatment conditions.](image)

C

| shACLY AKT | cisplatin |
|------------|-----------|
| - - +      | 24h       |
| + - +      | 48h       |
| + + +      | 72h       |

![Flow cytometry plots showing apoptosis analysis at 24h, 48h, and 72h for different treatment conditions.](image)

D

![Western blot images showing expression levels of various proteins for A2780/CDDP-NC-NC, A2780/CDDP-shACLY-NC, and A2780/CDDP-shACLY-AKT cells treated with 20μM cisplatin for 48h.](image)
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