CYLD and HER3 Regulate Platinum Resistance in Ovarian Cancer via Drug Efflux Pump ABCB1

Ye Zhang

Zhengzhou University

Xiao-Yu Jia

Zhengzhou University

Wei Wang

Zhengzhou University

Jian-Ge Qiu

Zhengzhou University

Wen-Jing Liu

Zhengzhou University

Chen Ding

Fudan University

Lin Wang

Zhengzhou University

Bing-Hua Jiang (✉ binghua.jiang@jefferson.edu)

Thomas Jefferson University <https://orcid.org/0000-0003-4526-2031>

Research Article

Keywords: Ovarian cancer, CYLD, Chemoresistance, HER3, ABCB1, Therapeutic target

Posted Date: February 14th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1330339/v1>

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Abstract

Background: Ovarian cancer (OC) is the most pathogenic gynecological malignant tumor in the world. Due to the difficulty of early diagnosis, most of patients developed chemo-resistance and recurrence during and after the chemotherapy.

Methods: CCK8 and flow cytometry analysis were used to analyze the drug sensitivity and apoptosis effect in drug resistant and their parental cell lines (A2780, A2780-DDP, OVCAR3, OVCAR3-DDP). Cells with CYLD knockdown or overexpression were used to test its role in DDP resistance. Patient tumor samples were used to study the clinical significance of CYLD and its related molecules. Flow cytometry analysis were used to test the drug efflux effects in CYLD knockdown cells. The drug combination method were used to investigate the drug synergistic effect of cisplatin combination with ABCB1 inhibitor or HER3 inhibitor. The protein and RNA levels were determined by Western blot analysis, PCR and immunoprecipitation. The *in vivo* experiments were used to verify the role of CYLD in drug resistance of ovarian cancer and tumor growth.

Results: we found levels of CYLD were markedly lower in ovarian DDP-resistant cancer cell lines than those of parental cell lines. CYLD knockdown in the parental cells alone was sufficient to induce DDP resistance through inhibiting cell apoptosis and promoting the drug efflux via inducing the expression of ABCB1. HER3 expression levels were much higher in the resistant cells, and HER3 was upstream mediator of CYLD suppression in the cells through STAT3 signaling. Moreover, overexpression of CYLD in the resistant cells alone was sufficient to reverse the resistant cells to become sensible to platinum-based treatment both *in vitro* and *in vivo*. Similar result showed that ABCB1 was functional downstream target of CYLD to regulate tumor growth and drug resistance both *in vitro* and *in vivo*.

Conclusions: In this study, the results demonstrated that CYLD suppression and higher HER3 expression caused cisplatin resistance and poor prognosis by inhibiting cell apoptosis and promoting the drug efflux in OC. Our findings showed that HER3/CYLD/ABCB1 is a new signaling pathway to regulate tumor growth and DDP resistance, which is potential new therapeutic target to overcome DDP resistance.

Background

Ovarian Cancer (OC) is the most lethal gynecological cancer. Due to difficult detection at early stage[1] and lack of significant clinical symptoms[2], 70% patients were diagnosed at an advanced stage(III-IV stage)[3], which also caused treatment failure. The most common histological type of ovarian cancer is epithelial ovarian carcinoma(EOC)[4], and the standard front-line treatment for EOC is constituted of surgery with the goal of no residual disease (R0) and platinum-based chemotherapy[5]. However, even after strict surgery and adequate chemotherapy, the survival rate of advanced ovarian cancer patients remains limited[6]. The prognosis of patients is closely related to their response to cisplatin treatment. Recently study showed that 19% of early ovarian cancers and 60%-85% of recurrent ovarian cancers will

eventually develop resistance to platinum[2]. Therefore, there is urgent need to understand the molecular mechanism of drug resistance in ovarian cancer.

Cisplatin (DDP), one of platinum drugs, exerts anticancer activity by forming Pt-DNA adducts and mediating DNA dysfunction[7, 8], eventually lead to cell apoptosis and exert anti-tumor effects[9]. There are several mechanisms are considered to relevant with DDP resistance, such as increased drug efflux, inactivation of apoptosis, increased DNA repair and alteration in drug target genes[10]. And the first two mechanisms accounted for about 80% cases of OC resistance through the analysis of GEO datasets [11]. Nevertheless, the initiated mechanism of cisplatin resistance is still not fully understood. More importantly, it is still unclear what combinational drug therapy could be an effective strategy to overcome the resistance in OC treatment.

HER3, a pseudokinase member of the EGFR family coded by the ERBB3 gene, plays an important role in cancer progression, despite its lack of intrinsic kinase activity[12, 13]. In our previous research, HER3 promoted angiogenesis in ovarian cancer[14] and positively regulated by reactive oxygen species (ROS) [15]. Recent studies have found that the increased expression of HER3 in breast cancer and lung cancer were closely related to TKi-resistance[16, 17]. However, the regulatory role of HER3 in other chemotherapy resistance was unclear yet. We found that HER3 was highly expressed in ovarian cancer cells, thus would like to test role of HER3 in drug resistance in this study.

The cylindromatosis gene (CYLD) is one member of lysine 63 deubiquitinases, which can specifically remove the k63 ubiquitin chain. CYLD was first discovered as a causative gene of familial cylindromatosis (an autosomal dominant hereditary disease), and caused CYLD cutaneous syndrome[18–20]. Further research showed that CYLD played an important role in tumorigenesis and development. CYLD had been reported as a tumor suppressor and associated with poor prognosis[18, 21, 22]. In addition, loss of CYLD directly or indirectly increased cell proliferation, inhibited cell apoptosis, induced immune response, abated inflammatory response, and regulates tumor development[23]. Furthermore, CYLD interacted with RIP1 to promote necroptosis[24]. However, role of CYLD in OC and platinum resistance is unclear yet.

In this study, we firstly identified that CYLD was downregulated in ovarian cancer, more interestingly, expression levels of CYLD were significantly decreased in cisplatin resistance cells (A2780-DDP, OVCAR3-DDP) when compared with their parental cells. Herein we addressed several important questions: (1) what is role of CYLD in regulating ovarian cancer resistance to DDP; (2) what is the underlying mechanism of CYLD in mediating DDP resistance; (3) what is the signal pathway that may be associated with CYLD expression and OC progression. (4) Whether there are some small molecular inhibitors or drugs could reverse the DDP resistance due to CYLD knockdown. The answers to these questions would provide new insights into a better understanding of the role and mechanism of CYLD in OC development and drug resistance and provide a potential therapeutic strategy to overcome resistance to DDP treatment in the future.

Methods

Clinical tumor samples

The 18 EOC tissues and 6 non-cancer (NC) tissues were obtained from the Biobank of the Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China). The ovarian cancer tissues had been collected and stored in the tissue bank from patients who did not receive immunotherapy and neoadjuvant chemotherapy before the surgery as previously described [25]. The patient information including names and other personal information was not known to the investigators. Based on the coded information, the EOC patients were staged according to FIGO classification[26], graded according to the World Health Organization standard[27]. The tumor tissues of patient Case 1 and Case 2 and NC samples (4 fibroid patients and 2 atypical hyperplasia of endometrium patients) were also obtained from the Biobank of the Affiliated Cancer Hospital of Zhengzhou University.

Immunohistochemistry

Histological sections of paraffin-embedded biopsies were derived from the tissues obtained from the Affiliated Cancer Hospital of Zhengzhou University from January 2017 to December 2020, including 33 EOC samples, 8 ovarian borderline epithelial tumor (LMP) samples, and 10 normal ovarian (or non-cancer, NC) samples. Immunohistochemistry was carried out as previously described [28]. Briefly, the samples were performed on 3- μ m-thick sections. After blocking with 10% normal goat serum, the sections were incubated with primary antibodies at 4 °C overnight. Then the sections were incubated with secondary antibody for 2h at room temperature.

Immunohistochemistry results were evaluated by the quality of the positive cells and described by immunoreactivity scores. Briefly, the density signals of positive cells were categorized into four grades: 0, negative; 1, weak; 2, moderate; and 3, strong, and categorized into two groups: negative (grade of 0 and 1), and positive (grade of 2 and 3). The percentages of the positive cells were categorized into four grades: 0 (0–3%) ; 1, (3–25%); 2, (26–50%); 3, (51–75%); and 4, (76–100%). The immunoreactivity scores of each field were determined using the following formula: intensity score $\times$ positive score. Then the score of one section was calculated using all fields' scores in the section, and categorized into two groups: low expression (scores of 0 to 6), and high expression (scores of 7 to 12)[29].

Cell culture

The human ovarian cancer cell lines Caov3, A2780, A2780/DDP, SKOV3, OVCAR3, OVCAR3/DDP, immortalized ovarian epithelial cell line IOSE386 and human embryonic kidney HEK 293T cell line were cultured in RPMI-1640 or DMEM medium (Gibco, California, USA), supplemented with 10% fetal bovine serum (Gibco, California, USA), penicillin(100U/ml) and streptomycin(100ng/ml). All cells were cultured at 37 °C with 5% CO₂ in incubator.

RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNAs of tissues and adherent cells were extracted using TRIzol reagent (Ambion, Texas, USA) according to the manufacturer's instruction, reverse transcription were performed using HiScript III RT SuperMix (Vazyme, Nanjing, China). The real-time PCR amplifications were performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) using QuantStudio5 system (Applied Biosystems, California, USA). GAPDH levels were used as an internal control. Real-time quantitative PCR was performed in triplicate. The primer sequences used for real-time quantitative PCR were listed in Supplemental Table 1.

Western blot

Total proteins extracted from tissues and adherent cells were separated by SDS-PAGE Gel Kit (Thermo Fisher Scientific, Waltham, USA) and transferred into poly-vinylidene difluoride (PVDF) membranes. Then the PVDF membranes were blocked with 5% non-fat milk for 2 h at room temperature and incubated with primary antibodies at 4°C overnight. After that the membranes were incubated with anti-rabbit/mouse IgG second antibody. The protein bands were detected with enhanced chemiluminescent substrate (Thermo Fisher scientific, Waltham, USA), as described previously[30]. The primary antibodies used in this study were listed in Supplemental Table 2.

The synergy of drug combination and quantitative assay

Cells were seeded into 96-well plates (5×10^3 per well), and treated with different doses of cisplatin, TX1-85-1 and Verapamil for 72h. Cell viability levels were analyzed by CCK-8 cell survival assay. The combined effects of drugs were analyzed by CompuSyn software (www.combosyn.com), and the drug dose-effects were calculated using the Median Effects methods. The results were quantitated according to the Chou-Talalay method using the fraction affected (Fa), and the combination index (CI). The quantitative assay staining signals were carried out and detected as described previously[31].

Tumor xenograft assay

Four to five-week-old female nude mice were obtained from GemPharmatech (Nanjing, Jiangsu, China). The mice were bred in a standard laboratory environment and fed with sterilized food and water. The animal study protocol was approved by the Animal Ethics Committee of Zheng Zhou University.

Drug resistance test: The mice were divided into 2 groups randomly, each group has 13 female mice. The stable shNC or shCYLD OVCAR3 cells were resuspended at a density of 5×10^6 in mixture of Matrigel and serum-free RPMI1640, and injected subcutaneously in the armpit of nude mice. When the tumors were approximately $0.5 \times 0.5 \text{ cm}^2$ (length of vertical radial line) in size, the mice were injected with cisplatin (5mg/kg, HANSOH PHARMA, Jiangsu, China) in intraperitoneal daily for 5 days, and the tumor sizes were measured every day. The tumor volumes (V) were calculated as: $V = (\pi/6) \times [\text{Sum of two vertical diameter lines}/2]^3$. Mice (4 with shNC xenograft tumors and 4 with shCYLD tumors) in all 26 mice were divided to do the drug tolerance test, when the tumor volumes reached $15 \times 15 \text{ mm}^3$, the mice were sacrificed, and tumor tissues were excised and weighted. For the survival experiment, the other shNC (n=9) or shCYLD

(n=9) xenograft mice were analyzed to measure out the survival rate from cisplatin injection to death and data were recorded.

Drug resistance reversal test: 9 mice were divided into 3 groups randomly, each group has three female mice. The mice injected subcutaneously with A2780, A2780-DDP and A2780-DDP with CYLD overexpressed cells and resuspended at a density of 5×10^6 in the armpit as described previously. Then the mice were treated with cisplatin (5mg/kg, daily for 5 days), tumor volumes and weights were analyzed with the same methods as mentioned above.

Drug combination test: 12 mice injected with stable CYLD knockdown OVCAR3 cells resuspended at density of 5×10^6 in the armpit as described above. The mice were randomized into four groups and treated with the following regimens: vehicle alone (0.9% saline), DDP (3 mg/kg, intraperitoneally), Verapamil (25 mg/kg, intraperitoneally), and the combination of DDP and Verapamil daily for 5 days. Then the tumor volumes and weights of each group were analyzed with the same methods as above.

HE staining and IHC staining in tumors

Mice xenograft tumors were fixed in 4% paraformaldehyde for at least 24h. After dipped in paraffin, the tumor samples were cut into 3 μ m thick sections. Then Hematoxylin and Eosin (HE) were used to stain the sections, and IHC staining were carried out as described previously[32]. The anti-bodies against Ki-67, CYLD, Bcl-XL, Bcl-2, Bax, Bcl-3, ABCB1 (Sup table 3) were incubated with the sections at 1:200 concentration.

Statistical analysis

All data were calculated as means $\pm$ SD. and analyzed using t-test. Then the results from the statistical analysis were compared using GraphPad Prism 8 software. p-value at <0.05 was considered statistically significant.

Results

CYLD levels were significantly lower in ovarian cancer DDP resistant cell lines, and CYLD was an essential regulator to mediate DDP resistance.

To identify new mechanism of DDP resistance in ovarian cancer, we found that the mRNA and protein expression levels of tumor suppressor CYLD were significantly downregulated in DDP resistant cell lines (OVCAR3-DDP and A2780-DDP) compared to parental A2780 and OVCAR3 cells, expression levels of CYLD in 4 different OC cell lines (Caov3, SKOV3, A2780 and OVCAR3) were greatly decreased compared to human immortalized ovarian epithelial cell line (IOSE386) (Fig. 1A, 1B). These results showed that CYLD expression was altered in progression of OC cisplatin resistance, but whether it is an initiating factor of drug resistance still needed to be investigated. To test whether CYLD suppression is sufficient in affecting the resistance, we showed that CYLD knockdown in OVCAR3 cells became more resistance to

DDP treatment compared to its control cells by CCK8 assay (Fig. 1C), and similar results from A2780 cells were obtained which was consistent with OVCAR3 cells (Fig. 1D). Additionally, the CYLD overexpressed OVCAR3-DDP/A2780-DDP cells were established, and overexpression of CYLD suggested to be sufficient to increase cell sensitivity to DDP compared to control group, making the DDP sensitive cell lines similar to OVCAR3 and A2780 to DDP treatment (Fig. 1E, 1F). These findings suggested that CYLD is an essential regulator in DDP resistance of the cells.

CYLD silencing was associated with resistance to DDP treatment in clinical ovarian cancer tissues.

Resistance to DDP treatment is a major clinical challenge in ovarian cancer therapy. Here, using computed tomography (CT) analysis with tumor tissues of 2 clinical patients, we firstly investigated that CYLD expression was attenuated in ovarian cancer patient tissues when the cancer became DDP resistance. Case 1: A 57-year-old subject underwent comprehensive staging surgery (including hysterectomy, lymphadenectomy and partial colectomy) in June 2018. Postoperative finding confirmed the diagnosis of phase IIIC high-grade serous ovarian carcinoma. Then the patient was administered six cycle chemotherapy of docetaxel and nedaplatin from June to November 2018. According to RECIST v1.1, complete response was achieved (Fig. 2A, left panel). Then regular review was checked by serum biomarker and imaging examination every 2 months (Fig. 2C). Two months later, serum biomarker Ca125 was found to be raised, but no radiographic evidence. Then the progress note of this patient showed no any treatment at this period. Four months after treatment, there was pelvic shadow in CT (Fig. 2A, right panel), and with further elevated Ca125, which both suggesting platinum resistance recurrence (Fig. 2C). Case 2: A 46-year-old patient was hospitalized for ascites. After comprehensive staging surgery, she was diagnosed of phase IIIC high-grade serous ovarian carcinoma, and started treatments of docetaxel and carboplatin in June 2018. Six cycle chemotherapy later, the patient achieved complete response according to RECIST v1.1 (Fig. 2B, left panel), and regular reviewed every 2 months (Fig. 2C). Until now, there has been no recurrence in these three years. The CT images in right panel of Fig. 1B showed the imaging results at six months after treatment. Immuno-histochemistry staining for CYLD of these tumor tissues were conducted using the surgical specimens. And the CYLD expression level showed negative in patient with platinum resistance recurrence, but positive in platinum sensitive case 1 patient (Fig. 2D). Additionally, the mRNA expression levels of CYLD in Case 1 and Case 2 also confirmed that CYLD expression deficiency in platinum resistance ovarian cancer tissues (Fig. 2E).

CYLD knockdown inhibited cell apoptosis activities, while force expression of CYLD promoted ovarian cancer cells' apoptosis and increased cell drug sensitivity.

To evaluate whether CYLD regulated DDP resistance through apoptosis, the apoptosis rates were analyzed in OVCAR3 and A2780 cells treated with 5 μ M DDP or PBS for 48h, and the percentage of apoptotic cells in CYLD knockdown group (shCYLD#1: 18.3602 $\pm$ 0.1521; shCYLD#2: 22.1101 $\pm$ 0.5478) significantly decreased compared to that in the control group (shNC: 43.8950 $\pm$ 0.2750) with DDP treatment (Fig. 3A). Consistent with these findings, CYLD knockdown in A2780 cells showed decreased apoptosis activity (Supplemental Fig. 1A). These results indicated that CYLD may regulate DDP

resistance through cell apoptosis. In seeking the downstream apoptosis-related proteins of CYLD, our results suggested that the protein expression levels of pro-apoptosis factor Bax were downregulated in CYLD knockdown group, and the anti-apoptotic proteins Bcl-XL and Bcl-2 were upregulated (Fig. 3B, 3C). In addition, the percentage of apoptosis in A2780-DDP with overexpressed CYLD group (62.0300 ± 5.3606) was remarkably higher than that of A2780-DDP (13.4906 ± 0.4704), and the result was similar to A2780 (96.5807 ± 0.7804) (Fig. 3D, 3E). Further study suggested that compared to OVCAR3-DDP/A2780-DDP, protein expression levels of Bax were upregulated in CYLD overexpressed OVCAR3-DDP/A2780-DDP cells and OVCAR3/A2780 cells, while the protein expression levels of Bcl-XL were downregulated, respectively (Fig. 3F). These results showed that CYLD mediated drug resistance through apoptosis and Bax/Bcl-XL pathway.

CYLD was significantly downregulated in ovarian cancer patient tissues, and lower CYLD expression levels were associated with poor prognosis.

The expression levels of CYLD were tested in 10 non-cancer ovarian samples (NC) and 8 ovarian borderline epithelial tumor samples (LMP) and 33 ovarian cancer samples (EOC) by IHC as showed in Fig. 4A. The EOC patients showed lower CYLD positive expression rate (22% vs 75%, 22% vs 83%) and lower IHC scores (2.0909 ± 2.0112 vs 5.5 ± 1.9272 , 2.0909 ± 2.0112 vs 7.3 ± 2.2632) compared to LMP and NC groups (Fig. 4B, 4C). The CYLD expression levels were also analyzed using TCGA database, were showed to be downregulated in ovarian cancer tissues and correlated with clinical advanced stages (Supplemental Fig. 2A, 2B). Furthermore, the protein and mRNA expression levels of CYLD were tested in patient tumor samples (NC=6, EOC=18) using the tissues obtained from the tissue bank. The expression levels of CYLD in NC group (protein: 0.8836 ± 0.2426 , RNA: 1.2343 ± 0.2326) were much higher than those in EOC group (protein: 0.553 ± 0.1055 , RNA: 0.8055 ± 0.2840) (Fig. 4D-4F). Additionally, the protein and mRNA expression levels of CYLD from the same patient samples showed highly correlation ($R=0.8463$, $p=0.0005$) (Fig. 4G). And the patients with lower CYLD expression levels had unfavorable outcomes compared to those with higher CYLD expression level group ($HR=5.373$, $p=0.0316$) (Fig. 4H). Lower CYLD expression levels were associated with poor survival rate by using Kaplan-Meier Plotter analysis (Supplemental Fig. 2C). These findings suggested that CYLD is an essential molecule in mediating drug resistance, and the patients with lower CYLD expression had poor overall survival in the patient cohort.

HER3 was identified to negatively regulate CYLD expression via phosphorylation of STAT3.

To further investigate the molecular mechanisms in CYLD regulation, the mRNA expression levels of the ERBB family (EGFR, HER2, HER3, HER4) were detected in patient samples. There was a significant negatively relationship between the expression levels of HER3 and CYLD ($R=-0.4333$, $P=0.0389$, Fig. 5A), while other ERBB family members had no significant correlations (EGFR: $R=0.3670$ $P=0.0777$; HER2: $R=0.2378$ $P=0.2631$; HER4: $R=0.2394$ $P=0.2599$; Supplemental Fig. 3A). Therefore, HER3 may be potential upstream regulator of CYLD in ovarian cancer. To further confirm the role of HER3 in regulation of CYLD, we found that the protein expression levels of CYLD were decreased in HER3 overexpressed OVCAR3/A2780 cells (OVCAR3-HER3: 0.4591 ± 0.1349 , A2780-HER3: 0.2756 ± 0.1011), compared to their

control cells (OVCAR3-Vector: 1 ± 0.1448 , A2780-Vector: 1 ± 0.1150) (Fig. 5B). In addition, the expression levels of CYLD were higher in HER3 knockout OVCAR3/A2780 cells (OVCAR3-HER3 KO: 3.9798 ± 0.2808 , A2780-HER3 KO: 3.4267 ± 0.5085) compared to control cells (OVCAR3-Vector: 1 ± 0.3207 , A2780-Vector: 1 ± 0.3865) (Fig. 5C). These findings further suggested that HER3 may negatively regulate CYLD expression. To examine the mechanism that how HER3 attenuates CYLD's expression in OC cells, HER3 overexpressed cells showed significantly induction in the expression levels of key intermediate signaling molecules p-STAT3(T705) compared to control cells (Fig. 5D), while other candidate molecules, p-c-Jun, p-p38 and p-Erk1/2, were showed no significant changes (Supplemental Fig. 3B). Moreover, the expression levels of p-STAT3 in HER3 knockout OVCAR3/A2780 cells was significantly decreased compared to control (Fig. 5D). Furthermore, the immunoprecipitation assay showed that HER3 had a direct interaction with CYLD (Fig. 5E). Thus, these results showed that HER3 could inhibited CYLD expression via phosphorylation of STAT3.

HER3 functioned as a crucial upstream regulator in DDP resistance of ovarian cancer and HER3 inhibitor facilitated DDP sensitivity.

To further confirm the role of HER3 in ovarian cancer drug resistance, the protein levels of HER3 in OVCAR-DDP and A2780-DDP cells were showed higher compared to its parental cells (Fig. 5F). The HER3 knockout cell lines showed more sensitive to drug treatment (Fig. 5G), while HER3 overexpressed cell lines found to be more resistant to DDP treatment (Fig. 5H). These results illustrated that HER3 played an important role in drug resistance of ovarian cancer cells.

In order to test whether HER3 inhibitor could improve the DDP sensitivity, the drug combination effect of HER3 inhibitor (TX1-85-1) and DDP in A2780-DDP cell line were conducted. To identify combination effect, Chou-Talalay method were used to detect the synergistic effect of DDP (0, 0.1, 0.3, 0.5, 0.7, 1, 3, 5 μ M) in combination with TX1-85-1 (0, 0.3, 0.7, 1, 3, 7 μ M). The Combination Index (CI) data of these two drugs was showed in Supplemental Fig. 3C. The Fraction Affected-combination index (Fa) analyzed the effects of the combination with different doses of TX1-85-1 and DDP (Fig. 5I). The synergistic effect of these two drugs was described in a Scatter plot, and the fractions affected-combination index (Fa-CI) plot were almost below 1 (Fig. 5J), that indicated that these two drugs had synergistic effect. The synergistic effect suggested that inhibition of HER3 improved drug sensitivity in DDP resistance OC cells. Then according to the CI results, we selected two drug concentrations (DDP 7 μ M, TX1-85-1 3 and 7 μ M), which was the best combination values in all CI data, for further apoptosis experiment analysis (Supplemental Fig. 3E). The percentages of apoptosis cells treated with DDP and TX1-85-1 group were significantly higher than those in the cells treated with each single drug (Fig. 5K). The same trends were also revealed in a different cell type, OVCAR3-DDP (Supplemental Fig. 3D, 3F, 3G). These results strongly demonstrated that HER3 promoted DDP resistance in OC cells, and HER3 inhibitor enhanced the drug sensitivity in DDP resistance OC cells.

Drug efflux pump protein ABCB1 was a potential downstream molecule of CYLD-in regulating DDP resistance.

Since CYLD knockdown promoted drug resistance in OC, but the mechanism of CYLD in affecting DDP resistance is unclear. Thus, we adopted the intracellular levels of Rhodamine 123, a fluorescent drug that can be excreted through drug transporters on the surface of cell membrane, and measured between CYLD knockdown group and control in OVCAR3 cells. After incubated with different concentrations of Rhodamine 123, the fluorescence intensity of CYLD knockdown group was higher than control group, and the difference was more remarkable at the concentration of 2 μ M (Fig. 6A). This result suggested that CYLD knockdown promoted drug to pump from intracellular. Then the fluorescence intensity of cells in different time point after incubated Rhodamine 123 were also measured (Supplemental Fig. 4A), which showed that the fluorescence intensities were significantly different in 12h group, while there was no difference after 16h treatment (Supplemental Fig. 4B). To examine which protein was the potential target of CYLD in ABC transporter superfamily, which may be involved in drug resistance, the protein expression levels of ABCB1 were significantly induced in CYLD knockdown OVCAR3 (shCYLD#1 3.8966 $\pm$ 0.6160; shCYLD#2 3.4024 $\pm$ 0.3618) and A2780 (shCYLD#1 4.0252 $\pm$ 0.4677; shCYLD#2 3.8090 $\pm$ 0.5398) compared with control cells (OVCAR3 shNC 1 $\pm$ 0.3271; A2780 shNC 1 $\pm$ 0.6219) (Fig. 6B). We also tested ABCC1, ABCG2 and ABCG9 levels in OVCAR3 cells, and the results did not show any difference (Fig. 6C,6D). These results showed that CYLD knockdown could increase the expression level of drug efflux protein, and ABCB1 was a downstream molecule of CYLD.

In addition, the protein expression levels of ABCB1 in OVCAR3-DDP/A2780-DDP cells were showed to be greatly higher than those in other two cells (OVCAR3-DDP/A2780-DDP with CYLD overexpressed and OVCAR3/A2780 cells) (Fig. 6E). Based on the above results, upregulation of ABCB1 by CYLD knockdown was a key for inducing drug resistance of, ABCB1 was a downstream mediator of CYLD in regulating DDP resistance.

CYLD knockdown cells increased DDP resistance by inhibiting apoptosis and inducing ABCB1 expression *in vivo*.

To investigate whether CYLD knockdown may promote ovarian tumor growth and DDP resistance, we established a xenograft model by BALB/C nude mice with CYLD-knockdown cells and its control cells: OVCAR3-shNC and OVCAR3-shCYLD (Fig. 7A). The results suggested that tumors developed from OVCAR3-shCYLD group grew faster than tumors developed from the control group after injection of DDP (Fig. 7B, 7C, 7D). Furthermore, the survival times of the mice with OVCAR3-shCYLD cells were remarkably shorter than those of the mice in the control group (Fig. 7E). These findings demonstrated that CYLD knockdown promoted DDP resistance of ovarian tumors with shorter survival time. The tumor samples developed from OVCAR3-shCYLD group showed increasing expression levels of Ki-67, Bcl-XL, ABCB1, but decreasing expression level of Bax (Fig. 7F,7G). Ki67 is a proliferation marker, the results suggested the growth of tumors with CYLD knockdown cells was not much influenced by DDP *in vivo*. And Bcl-XL and Bax are vital proteins in apoptosis pathway, these results showed that CYLD knockdown affected the apoptotic pathway and elevated ABCB1 expression which could promote drug resistance through drug efflux *in vivo*. These findings showed similar results of our experiments *in vitro*, that inhibition of CYLD in

ovarian cancer cells induced DDP resistance by regulating apoptosis pathway and ABCB1 expression *in vivo*.

Force expression of CYLD partially reversed DDP resistance, and dual treatment with DDP and ABCB1 inhibitor significantly inhibited tumorigenesis.

Based on the results above, we further established a xenograft model with CYLD-overexpressed A2780-DDP cells and its control cells: A2780 and A2780-DDP (Fig. 8A). The tumors from CYLD-overexpressed group and A2780 group grew slower than tumors from its control A2780-DDP group after treatment of DDP (Fig. 8B, 8C). These findings suggested that overexpressed CYLD could partially reverse DDP resistance in OC.

We wondered whether ABCB1 inhibitor Verapamil could reverse DDP resistance caused by CYLD knockdown in OC. The synergistic effects of DDP (0, 0.1, 0.3, 0.5, 0.7, 1, 3, 5 μ M) in combination with Verapamil (0, 0.7, 1, 3, 7, 10 μ M) were detected in OVCAR3 cells. The results of the CI data of these two drugs were showed in Supplement Fig. 4C. The synergistic effect of these two drugs in OVCAR3 cells was also described in a Scatter plot (Fig. 8D), and the Fa-CI plot showed these two drugs had synergistic effect (the value was almost below 1) (Fig. 8E). Then according to the CI results, these two drugs' concentrations (DDP 3 μ M, Verapamil 3 μ M), which had the best combination values among all the CI data, were selected for further apoptosis experiment (Supplemental Fig. 4D). The apoptosis rate of this combinatorial group in OVCAR3 cells was significantly higher than each single group (Fig. 8F). We obtained similar results in A2780-shCYLD cells (Supplemental Fig. 4E-4H). To further confirm the synergistic effect of DDP and Verapamil, we established a xenograft model with OVCAR3-shCYLD cells. As shown in Fig. 8G-8I, treatment with DDP or verapamil alone did not effectively inhibit tumor growth, while co-treatment with these two drugs inhibited tumor growth. Furthermore, the mice body weights were similar in all groups, suggesting that the combination regimen at the indicated dose did not cause any toxicity in mice (Fig. 8J). All these findings evoked that CYLD forced expression increased the DDP sensitivity in resistance OC, and inhibition of downstream protein ABCB1 also reversed the DDP resistance caused by CYLD depletion(Fig. 8K).

Discussion

Platinum resistance is one of major challenges that seriously affect the prognosis of ovarian cancer patients[33]. The 5-year survival rate of platinum resistance patient is approximately 30%[34], which is significantly less than that of platinum sensitive patient[2]. Interestingly, we found that CYLD expression was greatly downregulated in cisplatin resistance OC, which is correlated with poor prognosis (Figs. 2, Fig. 4). This result was consistent to other drug resistance in different cancers[21, 22, 35, 36]. Therefore, we hypothesized that CYLD played an important role in platinum resistance of ovarian cancer. To test the hypothesis, we performed both gain- and loss-of-function studies and demonstrated that CYLD forced expression inhibited ovarian cancer cisplatin resistance by inducing apoptosis and apoptosis-related proteins. Overexpression of CYLD *in vivo* also reversed DDP sensitivity in cisplatin-resistant OC. These

results indicated that CYLD may be a potential target for the diagnosis and treatment of DDP resistance in ovarian cancer.

Platinum-resistance ovarian cancers are defined that the platinum-free interval (PFI) of patient, means the time between the last dose of platinum-based chemotherapy and cancer progression, less than 6 months[37]. Previous studies of platinum resistance mostly focused on mediate apoptosis related pathway, such as the expression of “caspase” molecules inhibited[38, 39] or “Bcl-2” family molecules promoted[40, 41]. We observed higher expression of HER3 in ovarian resistant cancer cells. Since HER3 showed no intrinsic tyrosine kinase properties unless it went under heterodimerization and formed dimers such as HER2-HER3 and HER1(EGFR)-HER3[42]. However, role of HER3 in DDP resistance was not known yet. Our group showed higher expression levels of HER3 in ovarian cancer were induced by ROS[15] which played an important role in tumor angiogenesis[14]. HER3 could activate the PI-3K/Akt, MEK/MAPK, Jak/Stat pathways, and Src kinase[17]. In the present study, we found that HER3 promoted cisplatin resistance in ovarian cancer (Fig. 6), which was consistent with the clinical observation[43]. More importantly, HER3 reduced CYLD expression by promoting STAT3 phosphorylation, rather than p38, c-Jun and Erk pathways, which is novel. Interestingly, we also discovered the combination therapy of HER3 inhibitor TX1-85-1 and DDP remarkably reduced the dosage of platinum conducted in ovarian cancer resistance cells, and showed a significant synergistic effect. HER3 inhibitor may be used to treat DDP resistance in ovarian cancer.

Unexpectedly, we found enhanced drug efflux in CYLD knockdown ovarian cancer cells by flow cytometer, which had not been reported in previous studies. Since shortage accumulation of anti-cancer agents caused by increasing drug efflux is an important mechanism in regulation of chemo-resistance[44], we hypothesized that CYLD mediated platinum resistance in OC by blocking the drug efflux. Through both gain- and loss-of-function studies, we determined that CYLD mediated drug resistance by downregulating a downstream molecule, ABCB1 expression. And the significant synergistic effect of combination therapy (ABCB1 inhibitor Verapamil and DDP) further confirmed our hypothesis in CYLD knockdown OC cells. Thus, the combination therapy will enhance the efficacy of cisplatin, especially in cisplatin resistance OC.

Conclusions

In summary, we firstly identified the CYLD was a novel target for the diagnosis and treatment in cisplatin resistance OC via the HER3/CYLD/ABCB1 axis. Most importantly, the drug combination (HER3 inhibitor and DDP) in drug-resistant OC and the drug combination (ABCB1 inhibitor and DDP) in cisplatin-resistance showed significant chemotherapy coordination. These results provide new evidence and direction to overcome chemotherapy resistance in ovarian cancer which warrants further study in the future.

Abbreviations

OC

Ovarian cancer
CYLD
Cylindromatosis
DDP
Cisplatin
IHC
Immunohistochemistry
CCK-8
Cell counting kit 8
TCGA
The Cancer Genome Atlas
GEO
Gene Expression Omnibus.

Declarations

Ethics approval and consent to participate

Animal experiments were approved by the Animal Ethics Committee of Zheng Zhou University.

Consent for publication

All authors consent for publication.

Availability of data and material

All data generated or analyzed during in this study are included in this published article.

Conflicts of interest

The authors declare that they have no competing interests.

Funding

This work was supported in part by Provincial Health Commission of Henan (SBGJ202003013 and LHGJ20190656).

Acknowledgements

Not applicable

Authors' contributions

YZ, LW and BHJ participated in the design of the study. YZ, XYJ, and WW performed the experiments and analyzed the data. JGQ, WJL, and CD performed the bioinformatics analysis and analyzed the data. YZ , LW and BHJ wrote the manuscript. All authors read and approved the manuscript.

References

- 1 Shen L, Xia M, Zhang Y, Luo H, Dong D, Sun L. Mitochondrial integration and ovarian cancer chemotherapy resistance. *Exp Cell Res* 2021; 401: 112549.
- 2 Munoz-Galvan S, Carnero A. Targeting Cancer Stem Cells to Overcome Therapy Resistance in Ovarian Cancer. *Cells* 2020; 9.
- 3 Lukanovic D, Herzog M, Kobal B, Cerne K. The contribution of copper efflux transporters ATP7A and ATP7B to chemoresistance and personalized medicine in ovarian cancer. *Biomed Pharmacother* 2020; 129: 110401.
- 4 Kroeger PT, Jr., Drapkin R. Pathogenesis and heterogeneity of ovarian cancer. *Curr Opin Obstet Gynecol* 2017; 29: 26-34.
- 5 Daly MB, Pilarski R, Yurgelun MB, Berry MP, Buys SS, Dickson P *et al*. NCCN Guidelines Insights: Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic, Version 1.2020. *J Natl Compr Canc Netw* 2020; 18: 380-391.
- 6 Grunewald T, Ledermann JA. Targeted Therapies for Ovarian Cancer. *Best Pract Res Clin Obstet Gynaecol* 2017; 41: 139-152.
- 7 Ghosh S. Cisplatin: The first metal based anticancer drug. *Bioorg Chem* 2019; 88: 102925.
- 8 Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O *et al*. Molecular mechanisms of cisplatin resistance. *Oncogene* 2012; 31: 1869-1883.
- 9 Tchounwou PB, Dasari S, Noubissi FK, Ray P, Kumar S. Advances in Our Understanding of the Molecular Mechanisms of Action of Cisplatin in Cancer Therapy. *J Exp Pharmacol* 2021; 13: 303-328.
- 10 Freimund AE, Beach JA, Christie EL, Bowtell DDL. Mechanisms of Drug Resistance in High-Grade Serous Ovarian Cancer. *Hematol Oncol Clin North Am* 2018; 32: 983-996.
- 11 Wu C, He L, Wei Q, Li Q, Jiang L, Zhao L *et al*. Bioinformatic profiling identifies a platinum-resistant-related risk signature for ovarian cancer. *Cancer Med* 2020; 9: 1242-1253.
- 12 Haikala HM, Janne PAJCcraojotAAfCR. Thirty Years of HER3: From Basic Biology to Therapeutic Interventions27: 3528-3539.

- 13 Kiavue N, Cabel L, Melaabi S, Bataillon G, Callens C, Lerebours F *et al.* ERBB3 mutations in cancer: biological aspects, prevalence and therapeutics. *Oncogene* 2020; 39: 487-502.
- 14 He J, Jing Y, Li W, Qian X, Xu Q, Li FS *et al.* Roles and mechanism of miR-199a and miR-125b in tumor angiogenesis. *PLoS One* 2013; 8: e56647.
- 15 He J, Xu Q, Jing Y, Agani F, Qian X, Carpenter R *et al.* Reactive oxygen species regulate ERBB2 and ERBB3 expression via miR-199a/125b and DNA methylation. *EMBO Rep* 2012; 13: 1116-1122.
- 16 Yonesaka K. HER2-/HER3-Targeting Antibody-Drug Conjugates for Treating Lung and Colorectal Cancers Resistant to EGFR Inhibitors. *Cancers (Basel)* 2021; 13.
- 17 Liu X, Liu S, Lyu H, Riker AI, Zhang Y, Liu B. Development of Effective Therapeutics Targeting HER3 for Cancer Treatment. *Biol Proced Online* 2019; 21: 5.
- 18 Cui Z, Kang H, Grandis JR, Johnson DE. CYLD Alterations in the Tumorigenesis and Progression of Human Papillomavirus-Associated Head and Neck Cancers. *Mol Cancer Res* 2021; 19: 14-24.
- 19 Yang Y, Zhou J. CYLD - a deubiquitylase that acts to fine-tune microtubule properties and functions. *J Cell Sci* 2016; 129: 2289-2295.
- 20 Lork M, Verhelst K, Beyaert R. CYLD, A20 and OTULIN deubiquitinases in NF-kappaB signaling and cell death: so similar, yet so different. *Cell Death Differ* 2017; 24: 1172-1183.
- 21 Suenaga N, Kuramitsu M, Komure K, Kanemaru A, Takano K, Ozeki K *et al.* Loss of Tumor Suppressor CYLD Expression Triggers Cisplatin Resistance in Oral Squamous Cell Carcinoma. *Int J Mol Sci* 2019; 20.
- 22 Zhao J, Wang X, Mi Z, Jiang X, Sun L, Zheng B *et al.* STAT3/miR-135b/NF-kappaB axis confers aggressiveness and unfavorable prognosis in non-small-cell lung cancer. *Cell Death Dis* 2021; 12: 493.
- 23 Delbridge AR, Valente LJ, Strasser A. The role of the apoptotic machinery in tumor suppression. *Cold Spring Harb Perspect Biol* 2012; 4.
- 24 Dondelinger Y, Darding M, Bertrand MJ, Walczak H. Poly-ubiquitination in TNFR1-mediated necroptosis. *Cell Mol Life Sci* 2016; 73: 2165-2176.
- 25 Liu WJ, Huang YX, Wang W, Zhang Y, Liu BJ, Qiu JG *et al.* NOX4 Signaling Mediates Cancer Development and Therapeutic Resistance through HER3 in Ovarian Cancer Cells. *Cells* 2021; 10.
- 26 Prat J, Oncology FCoG. FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: abridged republication. *J Gynecol Oncol* 2015; 26: 87-89.
- 27 Meinhold-Heerlein I, Fotopoulou C, Harter P, Kurzeder C, Mustea A, Wimberger P *et al.* The new WHO classification of ovarian, fallopian tube, and primary peritoneal cancer and its clinical implications. *Arch Gynecol Obstet* 2016; 293: 695-700.

- 28 Werner B, Yuwono N, Duggan J, Liu D, David C, Srirangan S *et al.* Cell-free DNA is abundant in ascites and represents a liquid biopsy of ovarian cancer. *Gynecol Oncol* 2021; 162: 720-727.
- 29 Ma HM, Cui N, Zheng PS. HOXA5 inhibits the proliferation and neoplasia of cervical cancer cells via downregulating the activity of the Wnt/beta-catenin pathway and transactivating TP53. *Cell Death Dis* 2020; 11: 420.
- 30 Qiu J, Li Q, Bell KA, Yao X, Du Y, Zhang E *et al.* Small-molecule inhibition of prostaglandin E receptor 2 impairs cyclooxygenase-associated malignant glioma growth. *Br J Pharmacol* 2019; 176: 1680-1699.
- 31 Xia ZK, Wang W, Qiu JG, Shi XN, Li HJ, Chen R *et al.* Discovery of a New CDK4/6 and PI3K/AKT Multiple Kinase Inhibitor Aminoquinol for the Treatment of Hepatocellular Carcinoma. *Front Pharmacol* 2021; 12: 691769.
- 32 Feng J, Dai W, Mao Y, Wu L, Li J, Chen K *et al.* Simvastatin re-sensitizes hepatocellular carcinoma cells to sorafenib by inhibiting HIF-1alpha/PPAR-gamma/PKM2-mediated glycolysis. *J Exp Clin Cancer Res* 2020; 39: 24.
- 33 He J, Yu JJ, Xu Q, Wang L, Zheng JZ, Liu LZ *et al.* Downregulation of ATG14 by EGR1-MIR152 sensitizes ovarian cancer cells to cisplatin-induced apoptosis by inhibiting cyto-protective autophagy. *Autophagy* 2015; 11: 373-384.
- 34 Hao L, Wang JM, Liu BQ, Yan J, Li C, Jiang JY *et al.* m6A-YTHDF1-mediated TRIM29 upregulation facilitates the stem cell-like phenotype of cisplatin-resistant ovarian cancer cells. *Biochim Biophys Acta Mol Cell Res* 2021; 1868: 118878.
- 35 Wang Z, Wang Q, Xu G, Meng N, Huang X, Jiang Z *et al.* The long noncoding RNA CRAL reverses cisplatin resistance via the miR-505/CYLD/AKT axis in human gastric cancer cells. *RNA Biol* 2020; 17: 1576-1589.
- 36 Welte S, Urbanik T, Elssner C, Kautz N, Koehler BC, Waldburger N *et al.* Nuclear expression of the deubiquitinase CYLD is associated with improved survival in human hepatocellular carcinoma. *PLoS One* 2014; 9: e110591.
- 37 Kumar S, Oien DB, Khurana A, Cliby W, Hartmann L, Chien J *et al.* Coiled-Coil and C2 Domain-Containing Protein 1A (CC2D1A) Promotes Chemotherapy Resistance in Ovarian Cancer. *Front Oncol* 2019; 9: 986.
- 38 Janson V, Johansson A, Grankvist K. Resistance to caspase-8 and -9 fragments in a malignant pleural mesothelioma cell line with acquired cisplatin-resistance. *Cell Death Dis* 2010; 1: e78.
- 39 Cui Z, Pu T, Zhang Y, Wang J, Zhao Y. Long non-coding RNA LINC00346 contributes to cisplatin resistance in nasopharyngeal carcinoma by repressing miR-342-5p. *Open Biol* 2020; 10: 190286.

- 40 Long X, Xiong W, Zeng X, Qi L, Cai Y, Mo M *et al.* Cancer-associated fibroblasts promote cisplatin resistance in bladder cancer cells by increasing IGF-1/ERbeta/Bcl-2 signalling. *Cell Death Dis* 2019; 10: 375.
- 41 Kuo KL, Liu SH, Lin WC, Hsu FS, Chow PM, Chang YW *et al.* Trifluoperazine, an Antipsychotic Drug, Effectively Reduces Drug Resistance in Cisplatin-Resistant Urothelial Carcinoma Cells via Suppressing Bcl-xL: An In Vitro and In Vivo Study. *Int J Mol Sci* 2019; 20.
- 42 Molavipordanjani S, Hosseinimehr SJ. The Radiolabeled HER3 Targeting Molecules for Tumor Imaging. *Iran J Pharm Res* 2021; 20: 141-152.
- 43 McEvoy LM, O'Toole SA, Spillane CD, Martin CM, Gallagher MF, Stordal B *et al.* Identifying novel hypoxia-associated markers of chemoresistance in ovarian cancer. *BMC Cancer* 2015; 15: 547.
- 44 Zhai J, Shen J, Xie G, Wu J, He M, Gao L *et al.* Cancer-associated fibroblasts-derived IL-8 mediates resistance to cisplatin in human gastric cancer. *Cancer Lett* 2019; 454: 37-43.

Figures

Figure 1

CYLD levels were significantly lower in ovarian cancer DDP resistant cell lines, and CYLD was an essential regulator to mediate DDP resistance.

(A). The expression levels of CYLD in OVCAR3/OVCAR3-DDP and A2780/A2780-DDP were detected by western blot. (B). The mRNA expression levels of CYLD in human immortalized ovarian cell line (IOSE386), 4 human ovarian cancer cell lines (Caov3, SKOV3, A2780 and OVCAR3) and 2 DDP resistance

ovarian cancer cell lines (A2780-DDP and OVCAR3-DDP) were detected by qRT-PCR. (C, D). CYLD expression levels in stable CYLD knockdown OVCAR3 or A2780 cell lines were detected by Western blot, and the DDP sensitivity levels were detected by CCK8 assay in CYLD knockdown cells. (E, F). CYLD protein expression levels in indicated cell lines were tested by Western blot, and the DDP sensitivity levels in these three cell lines were measured using CCK8 kit. Data were statistically analyzed with Student's t-test and values are shown as mean $\pm$ SD. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Figure 2

CYLD silencing was associated with increased resistance to DDP treatment in clinical ovarian cancer samples.

(A, B). CT images of Case 1 and Case 2 ovarian cancer patients from Biobank at the time of treatment finished and 4/6 months after treatment. (C). The Ca125 levels of Case1 and Case2 patients at every

review time, the time 0 means the time of last treatment. (D). CYLD staining signals in tumor specimens from tumor tissues of these two patients. Magnification: 200×. (E). The mRNA expression levels of CYLD in tumor specimens from these two patients were analyzed.

Figure 3

CYLD knockdown inhibited cell apoptosis activities, while force expression of CYLD promoted cell apoptosis to further improve drug sensitivity.

(A). Incubated with 5 μ M DDP or PBS for 48h, the apoptosis rates of CYLD knockdown OVCAR3 cells and control cells were detected by flow cytometry, and the apoptosis rates were illustrated by the percentage sum of early and late apoptosis. (B). The apoptosis-related proteins were detected by Western blot. (C). The levels of apoptosis-relative proteins in CYLD knockdown cells and its control cells were quantified by densitometry and shown in a scatter plot. (D). After incubated with DDP for 48h, the apoptosis rates of A2780/A2780-DDP/A2780-DDP with CYLD overexpressed cell lines were detected by Flow cytometry analysis, (E). the apoptosis rates were illustrated by the percentage of early and late apoptosis. (F). The apoptosis-related proteins were also detected by Western bolt. Data were statistically analyzed with Student's t-test and values are shown as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4

CYLD expression was significantly downregulated in ovarian cancer tissues from different patients, and lower CYLD expression levels were associated with poor prognosis.

(A). Immunohistochemical staining signals of CYLD in clinical tumor samples, including normal ovarian tissues (n=10), Ovarian borderline epithelial tumor tissues (n=8), and ovarian cancer tissues (n=33),

original magnification, 200× or 400×. (B). The IHC staining intensity of CYLD were classified into negative (-~+) and positive (++~+++), and showed by proportion. (C). The immunoreactivity plots showed the IHC scores obtained for the staining signals of CYLD in different ovarian lesion samples. (D). CYLD protein expression levels were detected by Western blot in six NC samples and six EOC samples selected from tissue bank. β -actin levels were used as an internal loading control. (E). The levels of CYLD protein expression were quantified by densitometry and shown in a scatter plot. (F). Relative mRNA expression levels of CYLD by qRT-PCR in clinical tumor samples, including NC tissues (n=6) and EOC tissues (n=18) selected from tissue bank. (G). The correlations of mRNA and protein expression levels of CYLD were analyzed by comparing both mRNA and protein expression levels in the same patient tumor samples. (H). The survival time of the patient selected from tissue bank was analyzed by Kaplan-Meier. Data were statistically analyzed with Student's t-test and values are shown as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5

HER3 functioned as a crucial upstream regulator in DDP resistance of ovarian cancer by negatively regulated CYLD expression levels in HER3 knockout or

(A). The correlations of CYLD and HER3 levels were measured by Spearman correlation analysis in ovarian cancer patient samples. (B, C). The CYLD expression levels were detected in HER3 knockout or

overexpressed OVCAR3 and A2780 cells by Western blot. (D). Western blot analysis showed that levels of p-STAT3 protein in HER3 knockout or overexpressed OVCAR3 and A2780 cells; (E). After overexpression of HER3 in HEK293T cells, the direct interaction of HER3 and CYLD was detected and analyzed by immunoprecipitation assay. (F). The HER3 protein expression of OVCAR3-DDP/A2780-DDP cells were tested by Western blot. (G). The DDP sensitivity levels of HER3 knockout A2780/OVCAR3 cells were measured by CCK8 assay. (H). The DDP sensitivity levels of HER3 overexpressed A2780/OVCAR3 cells were measured by CCK8 assay. (I,J). The drug combination analysis of DDP and TX1-85-1 (HER3 inhibitor) in A2780-DDP cells were analyzed using CompuSyn software. The dose-effect curve and Fa-CI plot were shown. DDP (7 μ M) and TX1-85-1 (3 μ M and 7 μ M) resulted in CI value of 0.7494 and 0.8557. (K). The cell apoptosis of A2780-DDP cells co-treated by DDP (7 μ M) and TX1-85-1 (3 μ M and 7 μ M) were detected by Flow cytometry analysis, the apoptosis rates were illustrated by the percentage sum of early and late apoptosis. Data were statistically analyzed with Student's t-test and values are shown as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6

Drug efflux pump protein ABCB1 was an important downstream molecule in DDP resistance.

(A). OVCAR3-shCYLD1 cells were incubated with PBS for 12 hours at 37°C after pre-treated with 2µM or 5µM Rhodamine 123 for 2 hours, then measured the accumulation of Rhodamine 123 in cells by FCM. The quantified data were shown. (B). The quantitative of ABCB1 expression levels were illustrated by

densitometry and shown in a histogram. (C, D). The expression levels of ABC family proteins (ABCB9, ABCC1 and ABCG2) in OVCAR3-shCYLD cells and its control cells were detected by Western blot. (E). The ABCB1 protein expression levels in CYLD knockdown cells and OVCAR3/OVCAR3-DDP/OVCAR3-DDP with CYLD overexpressed stable cell lines were detected by Western blot. Data were statistically analyzed with Student's t-test and values are shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7

CYLD depleted ovarian cancer cells induced DDP resistance by regulating apoptosis and ABCB1 *in vivo*.

(A, B). The OVCAR3-shCYLD cells or OVCAR3-shNC cells were subcutaneous injected into the both sides of the armpit of BALB/C nude mice, and injected DDP(5mg/Kg) every day intraperitoneally for 5 days; then measured the tumor volumes every day. The quantified results and graph were shown. The tumor tissues of nude mice were presented. (C, D, E). The tumor growth curve, tumor weight and total survival curve were shown. (F). The expression levels of CYLD, Ki67, Bcl-XL, Bax and ABCB1 in animal tumor tissues were detected by immunohistochemical staining. (G). The quantified levels of immunohistochemical data were illustrated by IHC score, and shown in a histogram. Data were statistically analyzed with Student's t-test and values are shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8

Force expression of CYLD partially reversed DDP resistance, and dual treatment with DDP and ABCB1 inhibitor significantly inhibited tumorigenesis *in vivo*.

(A). The tumor tissues of nude mice with A2780, A2780-DDP and A2780-DDP with CYLD overexpressed cells were presented. The mice were measured every day, after injected DDP (5mg/Kg) every day

intraperitoneally for 5 days. (B, C). The tumor growth curve and tumor weight were shown in quantified data and graphs. (D, E). The combined treatment analysis of DDP and Verapamil (ABCB1 inhibitor) using CompuSyn software. The dose-effect curve and Fa-CI plot were shown. DDP (3 μ M) and Verapamil (3 μ M) resulted in CI value of 0.3792. (F). Co-treatment the A2780-shCYLD cells with DDP and Verapamil, the cell apoptosis rates were detected by Flow cytometry. (G). BALB/C nude mice were used to establish Xenograft experiment with OVCAR3 shCYLD cells, and detected the tumor volumes every day, after injection of DDP (3mg/Kg), Verapamil(25mg/Kg) or DDP(3mg/Kg) and Verapamil(25mg/Kg) every day intraperitoneally for total 5 days. The quantified data and graph were shown. the tumor tissues of nude mice were presented. (H, I, J). The tumor growth curve, tumor weight and mice weight were shown. (K). A schematic diagram illustrated the mechanism that HER3/CYLD/ABCB1 is a new signaling pathway to regulate tumor growth and DDP resistance. Data were statistically analyzed with Student's t-test and values are shown as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001.

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