CD147 promotes cisplatin resistance in ovarian cancer by inhibiting FOXM1 degradation via PI3k/Akt-GSK3β pathway

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Abstract

CD147 is a transmembrane glycoprotein that is highly expressed in a number of human cancers, including ovarian cancer. The antibody drug Licartin®, which targets CD147, has been approved by the Chinese Food and Drug Administration (FDA) and entered clinical treatment. Some studies have shown that CD147 plays a role on drug resistance, but the molecular mechanism remains elusive. This study sought to investigate the role and mechanism of CD147 in cisplatin resistance of ovarian cancer. We found that CD147 regulated ubiquitination and degradation of FOXM1 by activating PI3k/Akt-GSK3β pathway in ovarian cancer cells and further regulated the expression of DNA damage repair genes. The CUT&Tag-seq data showed that FOXM1 can directly bind to the promoter regions of several DNA damage repair (DDR) genes such as BRIP1, RRM1, FEN1, RAD50, and PMS2, thereby regulating their transcription. The siRNA against CD147 decreased the expression of FOXM1 and DDR genes, attenuating cisplatin resistance of ovarian cancer in vivo and in vitro experiments. Our results showed a novel cisplatin-resistant mechanism and suggest that the combination of cisplatin with a CD147 suppression is a prospective treatment plan for ovarian cancer that is resistant to cisplatin.

Introduction

Among gynaecological malignancies, ovarian cancer is the primary cause of mortality, seriously endangering the health of women. In the absence of early detection methods, most patients are already at an advanced stage when they are discovered (according to the FIGO staging, most are at stage III or IV). According to the NCCN guidelines, the standard chemotherapy regimen for ovarian cancer is basically based on platinum and paclitaxel, with cisplatin making up the largest proportion. Cancer patients frequently get cisplatin, a platinum-based medication, as their first-line treatment, and DNA is thought to be one of cisplatin's primary targets. When cisplatin is aquatized inside of a cell, the platinum atom forms a covalent bond with the N7 position of purines. This causes about 65% GpG, 25% ApG 1,2, and 5–10% GpNpG 1,3 intra-strand crosslinks, which inhibit cell growth and trigger the DNA damage response [1].

An important factor in ovarian cancer recurrence and death is the gradual development of drug resistance in patients during treatment with cisplatin, which significantly impairs the effects of chemotherapy. The mechanisms of platinum resistance can be broken down into a number of broad biological processes, such as (1) control of drug entry, exit, accumulation, sequestration, and detoxification, (2) improved repair and tolerance of platinum-induced DNA damage, (3) changes in cell survival pathways, and (4) modifications in pleiotropic processes and pathways, such as TGF-signaling, MYC signaling, NF-B signaling, receptor tyrosine kinase signaling, etc.[2]. Unfortunately, the specific molecular mechanisms associated with cisplatin resistance remain unclear. As a consequence, it is essential to continue researching the causes of cisplatin resistance.

High tumor cell DNA repair ability is a red flag for probable treatment resistance in patients [3]. It is commonly recognized that the human body has at least a few different DNA repair mechanisms, including homologous recombination repair (HRR), mismatch repair (MMR), base excision repair (BER),
and nucleotide excision repair (NER)[3]. The primary method for identifying and repairing DNA adducts caused by cisplatin is the nucleotide excision repair pathway. The elimination of cisplatin-induced DNA adducts is known to be accomplished by two important nucleotide excision repair mechanisms, transcription-coupled repair (TCR) and global repair (GR). As a result, the NER pathway is essential for repairing platinum DNA adducts and may affect a person's susceptibility to platinum treatment[1].

Numerous studies have demonstrated that CD147 is intimately connected to the malignant degree of tumor and that it is overexpressed in a wide range of cancer tissues [4–9]. Its antibody drug has been approved by the Chinese regulatory agency NMPA (the China National Medical Products Administration, formerly known as CFDA and SFDA) and entered into clinical treatment [10, 11]. The expression of CD147, which is significantly more frequent in ovarian cancer tissues than in normal ovarian cells, is a predictor of both overall survival (OS) and progression-free survival (PFS) in women with ovarian cancer [12, 13]. Despite multiple studies indicating that CD147 is implicated in cisplatin resistance of various cancers such as lung cancer [14], bladder cancer [15], colorectal cancer [16], gastric cancer [17], hepatocellular carcinoma [18] and head and neck squamous cell carcinoma [19], etc. the mechanism remains unclear.

Existing studies have shown that CD147 increases gemcitabine resistance in pancreatic ductal adenocarcinoma [20] and radiation resistance in cervical cancer [21] by regulating DNA damage response and duplex rupture repair ability. Whether CD147 can promote cisplatin resistance in ovarian cancer by regulating DNA damage repair ability, scavenging DNA adducts induced by cisplatin, and promoting cisplatin resistance is therefore an important mechanism that remains to be investigated.

In this investigation, we discovered that CD147 participates in the DNA damage repair process by regulating protein degradation of transcriptional factor FOXM1, which is a novel mechanism of CD147 in regulating cisplatin resistance of ovarian cancer cells. Therefore, CD147 can be considered as a potential target for combined cisplatin therapy.

**Results**

**CD147 promotes ovarian cancer cells to resist cisplatin drugs**

To determine the effect of CD147 on cisplatin sensitivity in ovarian cancer cells, we first established PDX model. The results of HE and CA125 staining showed that the PDX model was successfully created (Fig. 1A and supplemental Fig. 1A). The PDX-cisplatin resistance model was then drawn up in accordance with the method described. Following treatment with different cisplatin concentrations, the tumour volume and weight of mice in the cisplatin-resistant model group showed no significant difference (Fig. 1B and supplemental Fig.1B), whereas tumor volume and weight decreased significantly in the cisplatin-sensitive group (Fig. 1C and supplemental Fig.1C), indicating that the PDX-cisplatin resistance model was successfully constructed. Western blot detected the expression of CD147 in cisplatin-sensitive and drug-resistant PDX models, and the results showed that CD147 was significantly higher in cisplatin-resistant PDX models than in sensitive PDX models (Fig. 1D). Next, we developed cisplatin-resistant cell lines of ovarian cancer cell types A2780 and SKOV3, and examined the levels of mRNA and protein expression of
It was found that cisplatin-resistant cells expressed significantly more CD147 mRNA and protein (Fig. 1E and F). To further verify the contribution of CD147 expression to ovarian cancer cells' resistance to cisplatin, we interfered with or overexpressed CD147 in the A2780 and SKOV3 cell lines, respectively. The results showed that the cell viability was decreased in ovarian cancer cells treated with cisplatin simultaneously knocking down CD147 but increased in CD147 overexpression group, which demonstrating CD147 expression level is related to susceptibility of cisplatin in ovarian cancer cells. (Fig. 1G).

Cisplatin causes DNA damage and cell death by creating adducts with DNA. Therefore, we evaluated how ovarian cancer cells respond to DNA damage and if CD147 can influence DNA damage repair. Then, after cisplatin therapy, we determined the amount of γH2AX by detecting double-stranded DNA breaks. H2AX serves as a stand-in for double-strand breakage. DNA breaks and γH2AX levels were discovered using the comet assay and western blotting assay. The findings shown that down-regulated CD147 expression greatly increased the expression of γH2AX following a 48-hour treatment with 5ug/ml cisplatin (Fig1H). Double-strand breaks increased in frequency (Fig1I and J).

The anti-cisplatin action of CD147 is completed by encouraging the expression of DNA damage repair genes

Available data demonstrate that tumor cells are given the capacity to withstand cisplatin-induced DNA damage when CD147 molecules are expressed. Therefore, we hypothesize that CD147 facilitates tumor cells' ability to repair DNA damage in order to fulfill its role in drug resistance. We reviewed the literature and discovered 19 molecules that are closely associated to DNA damage repair in ovarian cancer. Western blotting and RT-PCR results revealed that after CD147 expression was downregulated, the levels of BRIP1, EXO1, RRM1, FEN1, MSH6, PMS2, RAD50 and XRCC1 mRNA and protein were considerably reduced (Fig2A and B).

Next, we conducted an analysis using the database AnimalTFDB3 (hust.edu.cn) and discovered that the upstream promoter region of the aforementioned 8 molecules shared a same transcription factor binding site. Analysis revealed that this location served as the transcription factor FOXM1's binding site (Supplemental Fig. 2A). After reducing the expression of FOXM1, we next determined the mRNA and protein expression levels of these DDR genes. The outcomes demonstrated that, following interference with FOXM1 expression, the mRNA and protein levels of these molecules were dramatically downregulated (Fig. 2C and D). To confirm the transcriptional regulatory impact of FOXM1 on DDR genes in more detail, we performed cut & tag analysis. The findings revealed that the FOXM1 peak was significantly enriched in FOXM1 overexpressing cell lines in contrast to the control group and was mainly enriched near the transcription start site (Fig2E and Supplemental Fig2B), of which 45.52% were located within the promoter sequence (Fig2G Supplemental Fig2C). Comparison with the human genome annotation database (TxDb.Hsapiens.UCSC.hg38.knownGene), our data revealed that 15561 and 15325 genes with differential FOXM1 binding were present in cell lines A2780 and SKOV3, respectively, and a total of 7995 genes were present in both cell lines (Fig. 2F). 34 genes are involved in DNA repair
Among eight genes regulated by CD147, we found that the promoter regions of five genes, RAD50, RRM1, PMS2, EXO1 and BRIP1, had significantly increased binding to FOXM1 in both cell lines (Fig2H Supplemental Fig2D). We analyzed the peaks of FOXM1 and found multiple FOXM1 binding sites in the promoter regions of these genes (Supplemental Fig2F). Analysis of correlations between these five genes and FOXM1 revealed a favorable association, and RRM1 had the strongest correlation with FOXM1, followed by BRIP1, both exceeding 0.4 (Supplemental Fig2E). Therefore, Whether CD147 affects DDR gene by regulating FOXM1 is the next question we need to study.

**Downregulation of FOXM1 inhibits the anti-cisplatin effect of CD147**

To verify the aforementioned hypothesis, we first identified FOXM1 mRNA and protein expression in cisplatin-resistant cells, and the findings indicated that FOXM1 was significantly overexpressed in cisplatin-resistant cells (Fig3A and B). Further, by overexpressing FOXM1 while simultaneously knocking down CD147, we were able to evaluate the expression of DDR genes. The results of Western blotting and RT-PCR showed that overexpression of FOXM1 counterbalanced the effect on DDR genes after interference with CD147 (Fig3C and E). CCK8 assay results also showed that overexpression of FOXM1 can counteract the changes in cisplatin sensitivity induced by CD147 silencing (Fig3F). The DNA damage and its marker γH2AX were assayed in ovarian cancer cells treated with a cisplatin concentration of 5 ug/ml for 48 hours and overexpression of CD147 knocking down FOXM1 with its specific siRNA or small molecular inhibitor, respectively, or combined overexpression of CD147 and knocking down FOXM1. Results from Western blotting revealed that, in contrast to the NC group, the expression of γH2AX substantially increased after a reduction in the expression of FOXM1 molecules caused by siRNA interference or FOXM1-specific inhibitors, and conversely, the expression of γH2AX significantly decreased upon an increase in the expression of CD147, which was counterbalanced by a reduction in FOXM1 expression. (Fig.3D). The same conclusion was drawn from the results of cellular immunofluorescence (Fig. 3G) and the comet assay (Fig. 3H), in which a decrease in FOXM1 expression significantly increased the extent of DNA damage in tumor cells treated with the chemotherapeutic drug cisplatin, a process that could be abolished by increasing the expression of CD147.

**CD147 regulates FOXM1 content via the PI3k/Akt-GSK3β signaling pathway**

The above results preliminarily demonstrated that CD147 can decrease the expression of FOXM1, which in turn regulated the expression of downstream DDR genes to achieve its anti-cisplatin effect. Following that, we investigated the specific mechanism by which CD147 regulates FOXM1 expression. Studies have shown that PI3k/Akt is a distinct downstream signal for CD147, and GSK3β kinase has been shown to regulate ubiquitous degradation of FOXM1 in glioma. Therefore, we hypothesize that CD147 may regulate FOXM1 degradation via the PI3k/Akt-GSK3β pathway.

To test this assumption, we first performed a correlation analysis of molecular expression in 28 tissues obtained by immunohistochemistry. The findings demonstrated a favorable correlation of pAKT protein content with CD147 and FOXM1 in ovarian cancer, which was statistically significant (Fig. 4A and B). We further found that downregulation of CD147 in ovarian cancer cell lines significantly reduced the protein
levels of pAKT and FOXM1 and increased the expression of p-GSK3β, but did not alter the mRNA levels of FOXM1. (Fig4C and Fig3E).

By overexpressing CD147 and using an inhibitor of both AKT and GSK3β, we were able to determine if CD147 controlled FOXM1 through the AKT pathway. Western blot analysis revealed that following overexpression of CD147, the expression levels of pAKT and FOXM1 were dramatically raised, but the expression levels of p-GSK3β were significantly lowered. We also found that both the AKT inhibitor LY294002 and the GSK3β inhibitor LiCl reversed the effect of CD147 upregulation to some extent. (Fig. 4D, E).

**FOXM1 completes protein degradation via the ubiquitination pathway**

GSK3β was shown to degrade FOXM1 by phosphorylating FOXM1 and promoting its binding to ubiquitination in glioma cells[22]. Firstly, we investigated whether FOXM1 was degraded via the proteasomal pathway in ovarian cancer cells with treatment of a combination of MG132 and CHX. The results showed a significant decrease in FOXM1 protein after treatment with the protein synthesis inhibitor CHX, indicating that significant protein degradation occurred after inhibition of FOXM1 protein synthesis, a process that could be blocked by the proteasome inhibitor MG132 (Fig. 5A). We then overexpressed HA-Ubiquitin molecules in ovarian cancer cells, treated them with MG132, and performed Co-Immunoprecipitation (COIP) with the FOXM1 antibody and detected HA molecules in the immunoprecipitation complex of FOXM1. Our results showed that FOXM1 can bind to Ubiquitin molecules, which in turn means that FOXM1 could be ubiquitinated, resulting in protein degradation (Fig. 5B). In addition, the results of co-immunoprecipitation showed an interaction between FOXM1 and GSK3β (Fig. 5C). It is the same as reported in the literature that GSK3β promotes the degradation of FOXM1 protein by interacting with FOXM1[22].

To test the regulation of FOXM1 degradation by GSK3β, we treated ovarian cancer cells with the GSK3β inhibitors LiCl and CHX and found that FOXM1 was significantly downregulated in ChX-treated cells, whereas LiCl suppressed this process, suggesting that inhibition of GSK3β could significantly block the FOXM1 degradation pathway (Fig. 5D). Next, we found that overexpression of CD147 significantly increased FOXM1 expression in ChX-treated cells (Fig 5E). This indicated that CD147 can inhibit FOXM1 degradation. These results suggest that FOXM1 protein is degraded via the proteasome pathway regulated by GSK3β activity, and CD147 can also inhibit FOXM1 degradation.

**In vivo experiments, downregulation of CD147 promotes the therapeutic effect of cisplatin drugs**

To support the notion that CD147 contributes to cisplatin resistance in ovarian cancer, we established a subcutaneous tumor model of human ovarian cancer in nude mice to determine the influence of CD147 downregulation on the efficacy of cisplatin treatment in vivo. The experimental model was divided into two groups, the cisplatin monotherapy group and the CD147 siRNA combined cisplatin treatment group. The mice received an injection of cisplatin (2.0 mg/kg) and siRNA (2.5 mg/kg) every three days. Mice
were killed 21 days later, and tumor tissue was collected for histological analysis and RNA and protein extraction.

Tumor measurements and weight data showed that tumor growth was inhibited in the siRNA and cisplatin combination group compared with the cisplatin monotherapy group (Figure 6A-C). In addition, we examined the expression of CD147, FOXM1, EXO1, RAD50, RRM1, PMS2, and BRIP1 in the harvested tumors. RT-PCR the Western blot and IHC results showed that the siRNA combination group significantly decreased the expression of DDR genes, inhibited the level of DNA damage repair (Figure 6D-F), and increased the tumor sensitivity to cisplatin treatment compared with the cisplatin monotherapy group. These findings imply that the CD147 molecule may enhance the ability of cisplatin to treat ovarian cancer.

Collectively, our data show that CD147 promotes FOXM1 degradation via the PI3K/AKT/GSK3β pathway, regulates DDR gene expression, and promotes cisplatin resistance in ovarian cancer.

**Discussion**

Although many studies have shown that CD147 promotes the cisplatin resistance, the mechanism keeps unclear. The principle of cisplatin therapy is that it forms an adduct with DNA that causes DNA damage and cell death. Therefore, resistance to DNA damage is the key mechanism for cisplatin resistance. Studies have shown that CD147 can regulate the expression levels of drug transporters ABCB1 (MDR1) and ABCG2, suggesting that it is involved in the pre-damage mechanism of DNA. In this study, we investigated whether CD147 participates process of DNA repair. Our results reveal that CD147 regulates the ubiquitination and degradation of FOXM1, which in turn promotes the repair of DNA damage and acquire drug resistance in ovarian cancer cells.

We firstly searched the ovarian associated DNA repair molecules according to references and found 8 out of 19 molecules regulated by CD147, which strongly suggests that CD147 might involve repair of DNA damage caused by cisplatin. Next, we analysed binding sites of the transcriptional factors in promoters of all these DNA repair molecules and found that all them have FOXM1 binding sites. We therefore infered that CD147 might regulate these molecules by regulating FOXM1.

Generally highly expressed in a range of tumor tissues, FOXM1 is a transcription factor that controls the expression of cell cycle genes necessary for DNA replication and mitosis and is crucial for the emergence and development of malignant tumors. FOXM1 is critical in high-grade ovarian cancer and participates in a number of pathophysiological processes via activating the transcriptional expression of downstream molecules, including cell proliferation, cell cycle, cell differentiation, cell apoptosis, invasion, angiogenesis, and DNA damage repair, etc. [23] [24] [25, 26].

Numerous studies have documented the role of FOXM1 in DNA damage resistance, including the involvement of single-stranded MMR, BER and double-stranded NHEJ and HR repair. Using Cut&Tag technology we acquired 33 DNA repair molecules out of 13794 FOXM1 potential regulated genes.
Among them, 5 DDR molecules such as EXO1, RRM1, RADA50, BRIP1, PMS2 were common regulated by CD147 and FOXM1, which suggests that most regulatory DDR genes of CD147 might be completed by FOXM1. EXO1, a DNA damage repair exonuclease, participates in a number of DNA damage restoration processes, such as mismatch repair [27] and homologous recombination repair [28]. In cisplatin-resistant cell lines, EXO1 is extensively expressed, and through taking part in NHEJ[29], it contributes to cisplatin resistance in mammals. Other research has verified that FOXM1 can support DNA repair by directly controlling EXO1’s transcription, defending ovarian cancer cells from cisplatin-induced death[30].

Ribonucleotide reductase M1 (RRM1) is a transporter involved in DNA repair and synthesis. In 68 patients who had had radical surgery for non-small cell lung cancer, the association between RRM1 protein expression and the effectiveness of adjuvant treatment with gemcitabine/cisplatin was examined. The results showed that RRM1 had a significant effect on the treatment effect (P < 0.05), and they predicted that RRM1 protein could be a valuable predictor of adjuvant chemotherapy with gemcitabine/cisplatin in patients with non-small cell lung cancer[31].RAD50 is an ATP-regulated cross-linking protein that acts as a bridge in DNA double-strand damage and can promote DNA end recognition and processing by MRE11 exonuclease, initiating DNA damage repair[32]. Several studies have shown that polymorphisms in the MRN gene (MRE11-RAD50-NBS1) are linked to an increased risk of ovarian and breast cancer[33]. Silencing of RAD50 by RNA interference can significantly enhance the cytotoxic effect of cisplatin and sensitize breast cancer cells to cisplatin/paclitaxel combination therapy[34]. A DEAH helicase called BRIP1 interacts with the BRCT domain of BRCA1[35] and is an important factor in homologous recombination repair. Studies have shown that ectopic expression of BRIP1 in the cervical cancer cell line (HeLa) can considerably improve cisplatin's antitumor effectiveness and encourage apoptosis induced by cisplatin[36]. PMS2, a key DNA mismatch repair (MMR) molecule, serves as a marker of MMR inactivation and is involved in cisplatin adduct recognition and activation, and decreased expression of MMR protein may reduce the sensitivity of testicular germ cell tumors to cisplatin [37].Our study shows that these molecules are directly transcriptionally regulated by FOXM1, which affects the ability of ovarian cancer cells to repair DNA damage by influencing the expression of these molecules, thereby playing a crucial part in ovarian cancer's cisplatin resistance.

Studies have demonstrated that CD147 can activate the PI3k/Akt pathway, both in non-tumor cells[38] [39, 40] [41] [42] and tumor cells such as HCC, colon cancer [43] [44]. Studies on the proteomics of the membrane surface of drug-resistant breast cancer stem cells have shown that the expression of CD147 is significantly increased, and inhibition of its expression can simultaneously suppress the activities of AKT and GSK3β[45]. Other studies have demonstrated that CD147 upregulates β-catenin expression via activation of PI3k/Akt and inhibition of GSK3β. These documents reveal that GSK3β can be regulated by CD147 and AKT [46]. Our previous study have proved that CD147 can activate PI3k/Akt signaling in HO-8910 ovarian cell [13]. We also confirmed that FOXM1 protein is regulated by GSK3β in glioma cells. In gliomas, GSK3β phosphorylates the serine at position 474 of FOXM1b and promotes ubiquitination and degradation of FOXM1, preventing its binding to β-catenin, attenuating the ability of β-catenin to enter the nucleus, and ultimately reducing cancer stem cell activity [22]. In the current research, we found that CD147 can activate PI3k/Akt signaling and inhibit GSK3β in ovarian cancer cells A2780.
and SKOV3. Expression of FOXM1 was increased in ovarian cancer cells by overexpressing CD147 to activate PI3K/Akt signal but decreased by Akt inhibitors. Moreover, our study demonstrated that the proteasome inhibitor MG132 GSK3β inhibitor LiCl and overexpression CD147 can effectively block the degradation of FOXM1 when inhibiting protein synthesis by CHX, which indicates that CD147 can inhibit ubiquitination degradation of FOXM1.

In summary, CD147 was upregulated in cisplatin-resistant ovarian cancer tissues and cell lines and participates cisplatin resistance. Overexpression of CD147 may regulate protein degradation of FOXM1 via the PI3K/AKT/GSK3β pathway, thereby regulating the expression of DNA damage repair genes. In conclusion, CD147/PI3K/AKT/GSK3β/FOXM1 axis may be an important mechanism and therapeutic target of cisplatin resistance in ovarian cancer.

Materials And Methods

Establishing of PDX model of cisplatin resistance in ovarian cancer

Following the established PDX model for ovarian cancer, the PDX resistance model was constructed. A total of 3 P4 generation tumor models were taken, each inoculated with 50mm³ unilaterally and observed for 21 days. Cisplatin 5mg/Kg was administered to the tumor volume of approximately 400 mm³ once a week 3–4 times, then the drug was discontinued. The tumor was dissected and transplanted into new mice. After growing up, the tumor was treated with cisplatin. After 3–4 repeated runs, the tumor inhibition rate, the expression of CA125 and CD147 were determined in the drug-resistant PDX model and in the parental model.

Cell culture and transfection

The authenticated cell lines A2780 and SKOV3 were received from the Air Force Medical University (Xi’an, China). All cell lines were grown in RPMI-1640 medium (Biological Industries) containing 10% fetal bovine serum (BI) and 1% penicillin/streptomycin. Following the manufacturer's instructions, Lipofectamine 2000 (Invitrogen) was used to transiently transfect plasmids and siRNA. The siRNAs, which focused on human CD147 and FOXM1, were bought from Gene Pharma in Shanghai, China. The following are the siRNA sequences (5'-3'): siCD147(GUUCUUCGUGAGUUCCUCTT), siFOXM1(GCUGGGAUCAAGAUUAUUATT). The negative control siRNA, siFOXM1 and siCD147 were transfected into A2780 and SKOV3 cells using lipo2000 transfection reagent, and the knockdown effectiveness was assessed by Western blotting and RT-PCR.

Western blotting

RIPA lysis buffer (Biosharp, Hefei, China, BL504A) was used to create the cell extracts, which were then centrifuged at 12 000 g for 15 min at 4°C. The protein samples were then transferred to polyvinylidene fluoride membranes (Millipore Corporation, Billerica, MA, USA) after being separated using 10% sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocking with 5% skim milk for an hour at room temperature, the primary antibody was applied to the membranes for an overnight treatment at 4°C. Subsequently, the membranes were incubated with a secondary antibody conjugated with HRP (Proteintech, Wuhan China). Chemiluminescence imaging technology was used to scan the membrane (Vilber, France). The following primary antibodies were used: CD147 (11989-1-AP, Proteintech), FOXM1 (20459, Cell Signaling Technology), γH2AX (9718, Cell Signaling Technology), Alpha Tubulin (11224-1-AP, Proteintech), p53 (10442-1-AP, proteintech), AKT (9272, Cell Signaling Technology), GSK3β (12456, Cell Signaling Technology), p-AKT (4060, Cell Signaling Technology), p-GSK3β (5558, Cell Signaling Technology), XRCC1 (21468-1-AP, proteintech ), PMS2 (18164-1-AP, Proteintech), RRM1 (60073-2-lg, Proteintech), EXO1 (16253-1-AP, Proteintech), FEN1 (14768-1-AP, Proteintech), MSH6 (66172-1-lg, Proteintech), BRIP1 (24436-1-AP, Proteintech), RAD50 (ab124682, Abcam).

RNA isolation and quantitative real-time PCR (RT-PCR)

Using the E.Z.N.A. Total RNA Kit I, total RNA was extracted from cultivated cells, and complementary DNA (cDNA) was produced using the Prime Script RT reagent kit (TaKaRa Bio, Otsu, Japan). SYBR Green PCR Master Mix (TaKaRa Bio, Otsu, Japan) and an RT-PCR machine were used to perform quantitative RT-PCR (Applied Biosystems, Irvine, CA, USA). The primer sequences for qPCR are listed in Supplemental Table 1. The levels of mRNA expression were adjusted to GAPDH.

Immunofluorescence

When the cells had expanded by 50%, the extra culture media was thrown out. The cells were then rinsed with PBS, fixed with 4% paraformaldehyde for 20–30 min at room temperature, permeabilized for 5 min with 0.2% Triton X-100, then blocked for 30 min at room temperature with 5% BSA to prevent non-specific protein interactions. The cells were then exposed to the primary antibody (Cell Signaling Technology, USA) at a dilution of 1:200 for an overnight incubation at 4 oC, followed by an hour-long incubation with the secondary antibody (Proteintech, Wuhan, China) at room temperature and in the dark. The cells were then exposed to DAPI for a further 15 minutes. Under a fluorescent microscope, the fluorescence pictures were seen.

Cell viability assay

In 96-well plates, cells were sown and adhered for 24 hours. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) test kit (Bimake) in accordance with the manufacturer's instructions after 48 hours of treatment with various dosages of cisplatin. In a nutshell, cells in each well were treated for two hours at 37°C with 10 µl of the CCK8 working solution. An Epoch Microplate Reader was used to measure the absorbance of each well at 450 nm (BIO-TEK, VT, USA).

Immunoprecipitation assay

PBS was used to clean all cells, and lysis buffer was used to lyse them. The lysate was centrifuged at 12 000 g for 15 minutes at 4°C. The appropriate antibody was used to immunoprecipitate the protein. Thermo Fisher Scientific, USA) protein A/G magnetic beads that had been cleaned were treated with the
immunocomplex before being rinsed with lysis buffer. After that, Western blotting was used to evaluate the samples.

**Comet assay**

A single cell suspension was prepared and mixed with low melting point agarose (LM agarose) at 37°C. The agarose was coated on the pretreated glass slides and lysed at 4°C overnight. Then the glass slides were immersed in the electrophoresis solution for gel electrophoresis. After the LM agarose is completely dry, a fluorescent dye was used to stain then and the degree of DNA damage was observed under a fluorescence microscope.

**In vivo animal experiments**

Female BALB/C nude mice (5 weeks old) were purchased from Wei tong Li hua Experimental Animal Center (Beijing, China). Mice were kept on a 12-hour light-dark cycle at a temperature of 22°C, given water, and normal laboratory food, and given at least a week to adjust. Each nude mouse received a subcutaneous injection of drug-resistant A2780-DDP cells into the axilla of the right upper leg. Each mouse received an inoculation of 5x10^6 cells, and there were 6 animals per group. One group received cisplatin and the other received cisplatin combined with CD147 siRNA at 10 days following inoculation, when the tumor was detectable. Cisplatin and siRNA were administered at dosages of 2.0 mg/kg and 2.5 mg/kg, respectively. Every three days during three weeks, siRNA was injected locally into the tumor along with an intraperitoneal injection of cisplatin. Measurements of the tumor's size and tumor growth curves were made starting on day 6 following therapy. At each injection, the weight and size of the tumor were recorded. Mice were executed at the conclusion of the therapy. Using immunocytochemistry, RT-PCR, and Western blotting, the expression levels of CD147, FOXM1, EXO1, RAD50, RRM1, PSM2, and BRIP1 were determined.

**Immunohistochemistry**

To solubilize the antigen, the tissue fragments were deparaffinized, hydrated, and given a citric acid disodium hydrogen phosphate treatment. They were then treated with the main antibody and an HRP-conjugated secondary antibody after being blocked with regular goat serum. Slides were DAB stained, hematoxylin counterstained, and given 1% hydrochloric acid alcohol treatment for distinction. They were then mounted, dried, and dehydrated before being examined under a fluorescence microscope.

**CUT&Tag(Cleavage Under Target & Tagmentation)**

Cells were counted and combined to ConA beads. The surfactant digitonin was added to enhance cell permeability so that the subsequent target protein-specific antibodies and protein A-Tn5 can enter the cells. The cells were incubated with the specific primary antibody against FOXM1, and followed with the secondary antibody. The assembled protein A-Tn5 fusion protein was added to form a complex of protein A-Tn5, FOXM1 antibody and chromatin. The process was triggered by the injection of magnesium ions (Mg2+), which caused the chromatin to be broken at the protein binding site and the insertion of the NGS adapter DNA sequences at the same time. This resulted in library preparation and chromatin cleavage in
a single process. SDS and proteinase K were added to lyse the cells and terminate the reaction. Finally, the DNA fragments were extracted, purified, amplified and sequenced in high-throughput DNA Sequencing Platform(GeneMind Biosciences, China)

**Data analysis**

All bar charts and XY graphs were generated using GraphPad Prism 7 after normalizing and organizing the data in Microsoft Excel. Quantitative data are shown as mean ± SE. The Pearson/Spearman correlation coefficient test or the two-tailed Student's t-test was used to establish significance (P < 0.05).

**Declarations**

**Authors’ contributions**

Yu Li, Miao Wang, Yu Wang : study concept and design; Miao Wang, Lin Chen, Tian Fan, Lei Mou, Zhen Zhang, Lin Chen, Arshad Ali experimental studies; Miao Wang, Tian Fan, Yu Wang, Chunyu Zhu, Zhixian Li : collection and data analysis and manuscript writing; Yu Li, Hong Yang, Jingyao Dai : obtained funding.

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**COMPETING interests**

The authors declare no competing interests.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Animal experiments were approved by the Animal Management Rule of the Chinese Ministry of Health and were performed in accordance with the approved guidelines and experimental protocol of Northwestern Polytechnical University. All animal experiments were in conformity with the Guide for the Care and Use of Laboratory Animals(National Academies Press, 2011).

**ADDITIONAL INFORMATION**

Supplementary dates include Supplementary Fig. S1 and S2 and Table 1 and 2.

**References**


35. INVALID CITATION !!! [36].


Figures

Figure 2
CD147 completes its anti-cisplatin effect by promoting the expression of DDR genes. (A). Analysis of mRNA expression by RT-PCR of DDR genes POLQ, BRIP1, EXO1, MSH2, XPB, PTEN, RRM1, FEN1, CDK2, RAD51, MSH6, PMS2, RAD50, BRAD1, FANCC, FANCA, PARP1, ARI1D1A, XRCC1 in A2780 and SKOV3 cells as indicated after siRNA interference with CD147 expression. (B). Western blot analysis for protein levels of DDR genes BRIP1, EX01, RRM1, FEN1, MSH6, PMS2, RAD50, XRCC1, in A2780 and SKOV3 cells as indicated after siRNA interference with CD147 expression. (C-D). RT-PCR and Western blot analysis for protein and mRNA expression of DDR genes BRIP1, EXO1, RRM1, FEN1, MSH6, PMS2, RAD50, XRCC1 in A2780 and SKOV3 cells as indicated after siRNA interference with FOXM1 expression. (E). Heatmap of normalized reads from genomic regions differentially bound by FOXM1 (left) and TSS enrichment (right) of differential FOXM1 motifs in SKOV3. FOXM1 peaks are ranked by intensity. (F). Venn diagram showing the overlap of annotated genes and DDR genes identified by differential FOXM1 binding peaks of A2780 and SKOV3. (G). Genome-wide distribution of FOXM1 binding peaks in SKOV3 and A2780 cells. (H). CUT&Tag signal at the typical target gene loci is tracked by genome browsers. The peak areas of FOXM1 on target-gene promoters are shown by the red rectangles.
Figure 3

Downregulation of FOXM1 inhibits the anti-cisplatin effect of CD147. (A-B): Western blot analysis (A) and RT-PCR (B) analysis for protein and mRNA expression of FOXM1 in A2780 and SKOV3 resistance cells. (C, E). Western blot analysis (C) and RT-PCR (E) analysis for protein and mRNA expression of DDR genes EXO1, PMS2, RRM1, BRIP1 and RAD50 in A2780 and SKOV3 cells as indicated after altered CD147 and FOXM1 expression. (D, G). Expression of the DNA damage marker γH2AX in cells treated for 48 hours.
with 3 ug/ml cisplatin and FDI-6 (10µM, 48h), siFOXM1, CD147 plasmid was detected by Western blotting (D) and cellular immunofluorescence (G). Scale bars= 100 µm. (F). Ovarian cancer cells were treated with CD147 siRNA or FOXM1 plasmid and different concentrations of cisplatin. Cell growth was detected by CCK8 assay. (H). Representative images and quantification of the Comet test. A2780 and SKOV3 cells were transfected with vector or siRNA and treated with DDP. The groups are as follows, siNC, siFOXM1, FOXM1 inhibitor FDI-6, CD147 overexpression, CD147 overexpression combined with FOXM1 siRNA or FDI-6. Cells were treated with 3ug/ml DDP for 48h. Left, typical Comet test pictures; right, quantification data. Scale bars: 20 µm.
CD147 regulated FOXM1 via the PI3k/Akt-GSK3β signaling pathway. (A) Representative patterns of IHC staining for CD147, p-AKT and FOXM1 in clinical samples of ovarian cancer. Scale bars: 50 μm. (B) Correlation analysis of p-AKT with CD147 and FOXM1 in clinical ovarian cancer tissues. Data source: IHC Quantitative Score. (C) Western blot analysis for protein of p-AKT, AKT, p53, GSK3β, p-GSK3β, FOXM1, p-FOX, and Tubulin in A2780 and SKOV3 cells as indicated after siRNA interference with CD147 expression. (D-E) Ovarian
cancer cells were pretreated with CD147 overexpression vector for 6h and then treated with LY294002 and LiCl for 48h. The expression of CD147, FOXM1, AKT, GSK3B, p-AKT, p-GSK3β by Western blotting (D). Amounts of CD147 and FOXM1 determined by densitometry of protein bands from two experiments. Tubulin was the loading control (E). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 5
FOXM1 completes protein degradation via the ubiquitination pathway. (A). CHX was applied to A2780 and SKOV3 cells either alone or in conjunction with MG132. Cells were harvested after various treatment durations to extract proteins and identify FOXM1 protein levels. (B). A2780/SKOV3 cells received co-transfection of HA-ubiquitin. Cells underwent a 6-hour treatment with 25 nM MG132 after 36 hours. IgG or FOXM1 antibody was used in IP on cell lysates, and then IB with anti-FOXM1 and anti-HA antibody was used. (C) Mouse anti-Flag antibody was used to perform IP on lysates from the SKOV3 and A2780 cells that were expressing Flag-FOXM1, and then IB was performed using the anti-GSK3β and FOXM1 antibodies. (D). LiCl (10 mM) and CHX (100 μg/ml) were applied to A2780 and SKOV3 cells for 48 hours, and proteins were then gathered to detect FOXM1 expression. (E). A2780 and SKOV3 cells overexpressed CD147 and the empty vector. The protein was extracted after the cells had been exposed to CHX (100 μg/ml) for a certain amount of time, and the presence of FOXM1 protein was determined.
In vivo experiments, downregulation of CD147 promotes the therapeutic effect of cisplatin drugs. (A). Representative images of the subcutaneous tumors from each group. (B). The tumor growth curves. (C). The average tumor weight of each group. (D-F). Western blot, RT-PCR and IHC analysis of CD147, FOXM1, EXO1, RAD50, RRM1, PMS2 and BRIP1 protein expression. Scale bars = 50 μm. (G) A schematic model (by Figdraw) of the role and mechanism of CD147 in ovarian cancer cisplatin resistance.
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