

Research Paper

# UCHL1 Is a Putative Tumor Suppressor in Ovarian Cancer Cells and Contributes to Cisplatin Resistance

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## Abstract

Ubiquitin carboxyl terminal hydrolase I (UCHL1) catalyzes the hydrolysis of COOH-terminal ubiquityl esters and amides. It has been reported as either an oncogene or a tumor suppressor in cancers. However, UCHL1's role in ovarian cancer is still unclear. Therefore, we conducted an analysis to understand the role of UCHL1 in ovarian cancer. Firstly, we detected UCHL1 promoter methylation status in 7 ovarian cancer cell lines. 4 of them with UCHL1 silencing showed heavy promoter methylation while the other 3 with relative high UCHL1 expression showed little promoter methylation. Then we reduced UCHL1 expression in ovarian cancer cell line A2780 and IGROV1 and found that inhibition of UCHL1 promoted cell proliferation by increasing cells in S phases of cell cycle. Knockdown of UCHL1 also reduced cell apoptosis and contributed to cisplatin resistance. Furthermore, the expression level of UCHL1 in several ovarian cancer cell lines correlated negatively with their cisplatin resistance levels. Microarray data revealed that UCHL1 related genes are enriched in apoptosis and cell death gene ontology (GO) terms. Several apoptosis related genes were increased after UCHL1 knockdown, including apoptosis regulator BCL2, BCL11A, AEN and XIAP. Furthermore, we identified up-regulation of Bcl-2 and pAKT as well as down-regulation of Bax in UCHL1 knockdown cells, while no significant alteration of p53 and AKT1 was found. This study provides a new and promising strategy to overcome cisplatin resistance in ovarian cancer via UCHL1 mediated pathways.

Key words: Ubiquitin carboxyl terminal hydrolase 1, tumor suppressor

## 1. Introduction

Ovarian cancer is a serious health problem for women all over the world. Due to non-obvious early symptoms, about 70% of all cases are diagnosed at advanced stages<sup>1</sup>. In the USA, estimated new cases of ovarian cancer in 2012 account for only 3% of all kinds of female cancer patients and its estimated death rate doubles, reaching 6%<sup>2</sup>, which is higher than other gynecological malignancies.

UCHL1, short for ubiquitin carboxyl-terminal hydrolase L1, is a member of ubiquitin carboxy ter-

minal hydrolase (UCH) family that catalyzes the hydrolysis of COOH-terminal ubiquityl esters and amides. UCHL1 was initially found to be expressed specifically in testis/ovary and brain<sup>3,4</sup>. UCHL1 mutations are related to neurodegenerative diseases such as Parkinson's diseases (PD) and Alzheimer disease (AD)<sup>5</sup>. UCHL1 has been reported as either an oncogene or a tumor suppressor gene. UCHL1 has been reported to be up-regulated in a variety of cancers. For example, over expression UCHL1 in lymphoma

strongly accelerated lymphomagenesis by boosting signaling through the AKT pathway via down regulation of the antagonistic phosphatase PHLPP1<sup>6</sup>. In non-small lung cancer cell line H157, UCHL1 promotes invasion by upstream activation of Akt<sup>7</sup>. In colorectal cancer, UCHL1 acts as an oncogene via activation of the beta-catenin/TCF pathway through its deubiquitinating activity<sup>8</sup>. In contrary, promoter hypermethylation that leads to silencing or low expression of UCHL1 were also found in several types of cancers, including nasopharyngeal carcinoma<sup>9</sup>, melanoma<sup>10</sup>, hepatocellular carcinoma<sup>11</sup>, esophageal squamous cell carcinoma<sup>12</sup>, renal cancer<sup>13</sup>, gastric cancer<sup>14</sup> and prostate cancer<sup>15</sup>.

Cisplatin is a common reagent used in chemotherapy for ovarian cancers with an initial response rate of about 70%, yet recurrence is common as patients often develop resistance to cisplatin therapy<sup>16</sup>. Proteins modulating cisplatin responses are of great importance to overcome cisplatin resistance. UCHL1 was a protein identified as differentially expressed proteins between the cisplatin-sensitive (A2780S) and cisplatin-resistant (A2780-CP) ovarian cancer cell lines<sup>17</sup>. However, the mechanism of how UCHL1 modulating cisplatin resistance has not been established. Here we describe our study in helping understanding the role of UCHL1 in chemotherapy response of ovarian cancers. We first showed that silencing of UCHL1 in ovarian cancer cell lines were caused by its promoter methylation and treatment of demethylation reagent could restore its expression. Furthermore, UCHL1 knockdown in ovarian cancer cell lines A2780 and IGROV1 promoted cell growth while causing reduction of apoptosis. Microarray analysis demonstrated that UCHL1 knockdown induced alterations in apoptosis and cell death. UCHL1 knockdown ovarian cancer cells (A2780-shUCHL1 and IGROV1-shUCHL1) became more resistant to cisplatin than their corresponding controls. Moreover, we found that UCHL1 expression level was negatively correlated with cisplatin resistance in ovarian cancer cells. We then detected up-regulation of Bcl-2 and down-regulation of Bax in UCHL1 knockdown cells, however, no significant alteration of p53 was detected. Also we detected up-regulation of phospho AKT. These results suggest that UCHL1 is mediator of apoptosis and targeting UCHL1 might be of great potential to overcome cisplatin resistance in ovarian cancer.

## 2. Materials and Methods

### 2.1 Cell lines and cell culture

Ovarian cancer cell lines A2780, A2780CP, SKOV3 and IGROV1 were kind gifts from Dr. Stephen

Collins from UC San Diego (CA, USA). ES-2, OVCAR3 and CAO3 were purchased from Cellbank, Chinese Academy of Sciences, Shanghai, China. ES-2 and SKOV3 were cultured in McCoy's 5A medium (Invitrogen, Carlsbad, CA) with 10% FBS, while other cell lines were cultured in RPMI 1640 (Invitrogen) medium supplemented with 10% FBS at 37 °C and 5% CO<sub>2</sub>.

### 2.2 Lentiviral vector and infection

UCHL1 was knocked down by pLKO.1-puro lentiviral Vector cloned with UCHL1-target shRNA (CCGGGTGTGAGCTTCAGATGGTGAAGCTCGAGTTCACCATCTGAAGCTCACACTTTTT), purchased from Sigma-Aldrich. A Non-Mammalian shRNA (SHC002V) was used as a negative control. The lentivirus or the control was added to A2780 and IGROV1 cells with 8 µg/ml (final concentration) hexadimethrine bromide (H9268, Sigma-Aldrich) for infection. 500ng/ml (final concentration) puromycin was added to select positive clones. Stable transfectants were isolated after 3 weeks selection.

### 2.3 MTT assay and Cytotoxicity assay

Growth was measured by MTT assay. Briefly, 3,000 cells were collected and seeded to each well of 96-well plate. After 1, 3, 5 days, MTT was added to cells (final concentration was 0.5mg/ml). After 4 hours incubation, medium was replaced by 100ul DMSO and OD value at 490nm was measured.

To measure cisplatin sensitivity, 8,000 cells were seeded to each well in 5 replicates of 96-well plate. Cells were treated with a series dose of cisplatin from 0 µg/ml to 16 µg/ml and 48 hours later MTT assay was performed to measure cell viability. To induce apoptosis to measure active caspase-3 protein level, 20µg/ml cisplatin (final concentration) was added and 16 hours later cells were harvested to perform western blot analysis.

### 2.4 RNA isolation and Real-time PCR

Total RNAs were extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. 2 µg RNA was reverse transcribed in a final volume of 25µl using M-MLV reverse transcriptase (Promega, USA). Real-time PCR were performed using SYBR-Green PCR kit (Takara, Japan) in Bio-RAD CFX96 Real-Time System. All measurements were performed in triplicate following the cycle scheme: 95 °C for 2 min, 40 cycles of 95 °C for 15s, 58 °C for 30s and 72°C for 15s. Primers for real-time PCR were designed as follows: UCHL1, forward, 5'- CCGAGATGCTGAACAAAG-3', and reverse, 5'-CAGAGACTCCTCTTCCAG-3'; BCL2, forward, 5'-ATAACGGAGGCTGGGATG-3', reverse, 5'-GCATGTGACTTCACTTGTG-3'; GAPDH, for-

ward, 5'-GGAGATTGTTGCCATCAACG-3', and reverse, 5'-TTGGTGGTGCAGGATGCATT-3'.

## 2.5 5-Aza-2'-deoxycytidine treatment

A2780CP cell line was treated with 10  $\mu$ M 5-Aza-dC (Sigma-Aldrich) for 5 days. Medium was replaced with fresh 5-Aza-dC every 24 hours.

## 2.6 DNA extraction, methylation specific PCR and bisulfite-modified DNA sequencing

Genomic DNA was extracted from cultured cells using DNeasy Blood & tissue Kit (Qiagen, Germany). Then genomic DNA was quantified and 250 ng of DNA was bisulfite-treated with EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA) according to the manufacturer's protocol. Methylation specific PCR (MSPCR) for UCHL1 was performed using Zymo Taq™ DNA polymerase (Zymo Research) with primers as follows: M, forward, 5'-TTTATTTGGTTCGCGATCGT TC-3', reverse, 5'-AAACTACATCTTCGCGAAACG-3'; U, forward, 5'-GTATTTA TTTGGTTGTGATTGTTT-3', reverse, 5'-CTTAAACTACATCTTCACAAAAC A-3'. The MSPCR was carried out following the polymerase's protocol with annealing temperature 57 °C.

For bisulfite-modified DNA sequencing, Zymo Taq™ DNA polymerase was used to amplify a fragment of the UCHL1 promoter CpG islands containing 34 CpG sites with primers as follows: forward, 5'-GTTTGGTTTTGTTTTT GTTTTTTTT-3', reverse, 5'-ACTTAATCCCTACCAACAACC-3'. The PCR cycle scheme was the same as MSPCR. PCR products were then cloned into pMD® 18-T Vector (Takara) and 6-8 colonies were randomly chosen for sequencing. Primers for both MSPCR and bisulfite PCR were designed by web tools MethPrimer (<http://www.urogene.org/cgi-bin/methprimer/metprimer.cgi>)<sup>18</sup>.

## 2.7 Flow cytometry analysis of cell cycle and apoptosis

For cell cycle assay, cells were cultured in 12-well plates to about 70% in reduced serum (1%) for 24 hours to get synchronized. Then cells were trypsinized and harvested, and washed with cold PBS. Next, cells were fixed with 80% cold ethanol over night at 4 °C. Fixed cells were centrifuged and resuspended in propidium iodide solution, and subjected to flow cytometry analysis after 30 minutes' incubation at 37°C.

For apoptosis analysis, cells (10,000/well) were cultured in 6-well plates to about 70% confluence then were cultured for 24 hours in reduced serum (1%). Then cells were collected and Annexin V-FITC/PI staining was performed according to standard pro-

ocols. After staining, cells were analyzed using BD FACS Array™ (Becton-Dickinson, San Jose, CA) according to manufacturer's guidelines.

## 2.8 Western blot

Protein was extracted using RIPA [50mM Tris(pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2mM sodium pyrophosphate, 25mM  $\beta$ -glycerophosphate, 1mM EDTA, 1mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 $\mu$ g/ml leupeptin] and concentration was measured with BCA Protein Assay Kit (23225, Thermo Scientific). Equal amounts of protein (20 $\mu$ g) were added to 12% SDS-PAGE gel for electrophoresis. After that, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% non-fat milk in TBST for 1 hour. Antibodies used were UCHL1 (PAB3903, Abnova), Bax (Epitomics), BCL2 (Epitomics), GAPDH (Epitomics). GAPDH was used as internal control.

## 2.9 Microarray and Gene Ontology analysis

To identify genes regulated by UCHL1, we performed a microarray assay using Affymetrix U133 plus 2 genechip (Affymetrix, Santa Clara, CA, USA). After RMA normalization, data were exported to the GeneSpring program (Agilent, Inc.) to filter out lowly expressed and unreliably detected genes with probe intensity <100. After that, genes with fold change <2 was furthered eliminated. Finally, Gene Ontology analysis was performed on line using the GoMiner program<sup>19</sup>.

## 2.10 Statistical analysis

All data were presented with mean  $\pm$  SD. Cell survival curves were fitted to the data points with nonlinear regression analysis using GraphPad Prism 5.0. Two-tailed Student's t-test was used to compare mean values.  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 UCHL1 expression was correlated with its promoter methylation and could be restored with 5-Aza-dC treatment

UCHL1 was reported frequently methylated in various cancers, including ovarian cancer<sup>20</sup>. To validate its promoter methylation status, we performed MS-PCR in 7 ovarian cancer cell lines (ES-2, A2780, IGROV1, OVCAR3, SKOV3, A2780CP and CAO3). We found that the methylation status of UCHL1 in the promoter region correlated negatively with UCHL1 expression: those cell lines with very low UCHL1 expression showed positive methylation while those with relatively high UCHL1 expression showed negative methylation (Fig.1A). We next performed bisul-

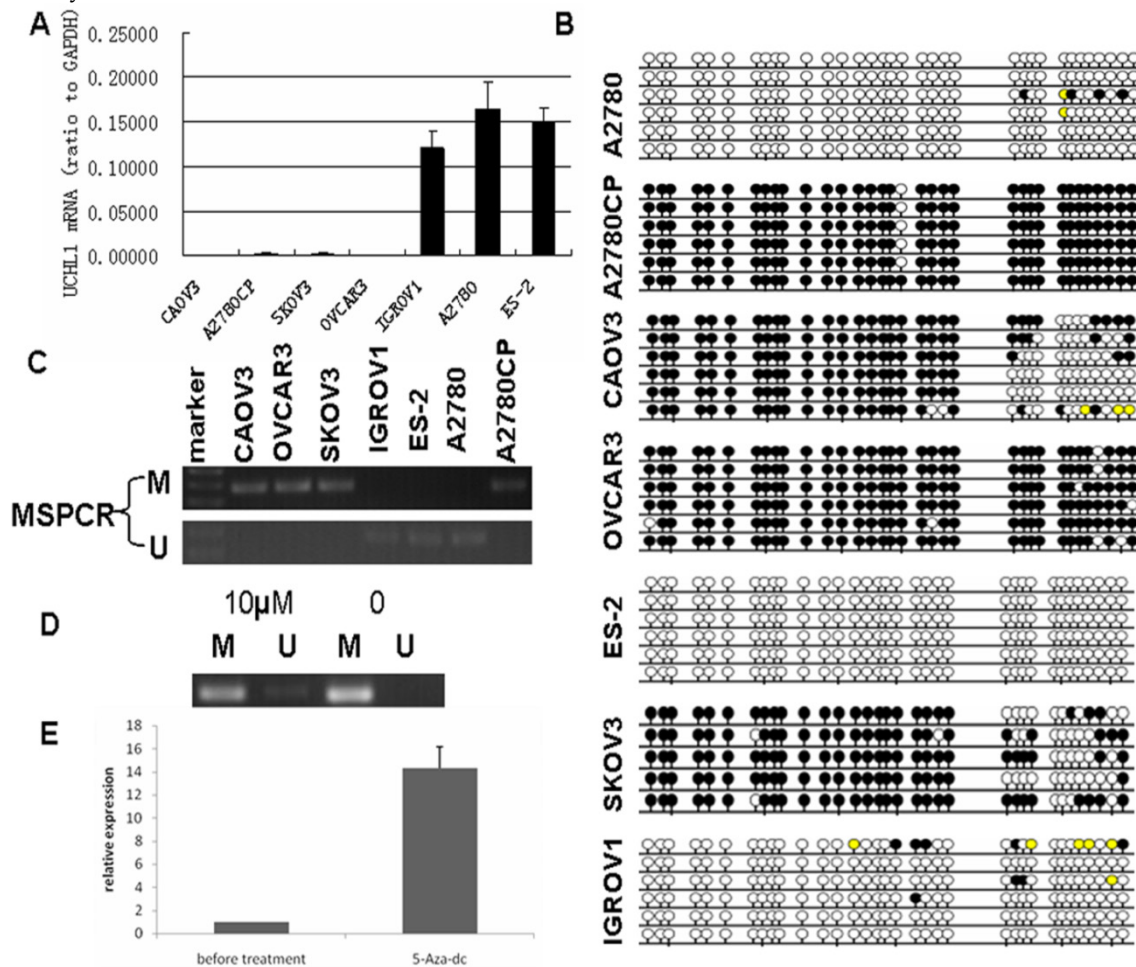


site genomic sequencing (BGS) of all 7 ovarian cancer cell lines (Fig. 1B). BGS data matched perfectly with MS-PCR results (Fig. 1C): The MS-PCR positive cell lines showed heavy promoter methylation while MS-PCR negative cell lines showed very weak or no promoter methylation (Fig.1B-C).

We then chose A2780CP, an isogenic cell line of A2780 that differ in cisplatin resistance, and treated A2780CP cells with demethylation reagent 5-Aza-dC for 5 days to confirm whether silencing of UCHL1 is caused by its promoter hypermethylation. After 5-Aza-dC treatment, A2780CP showed a weak band of unmethylation (Fig.1D). Furthermore, UCHL1 expression was significantly induced after 5-Aza-dC treatment by RT-PCR analysis (Fig.1E). These results suggest that hypermethylation of UCHL1 in promoter region is responsible for its transcriptional silencing.

### 3.2 Reduced expression of UCHL1 in A2780 and IGROV1 reduced cell apoptosis but increases cell proliferation

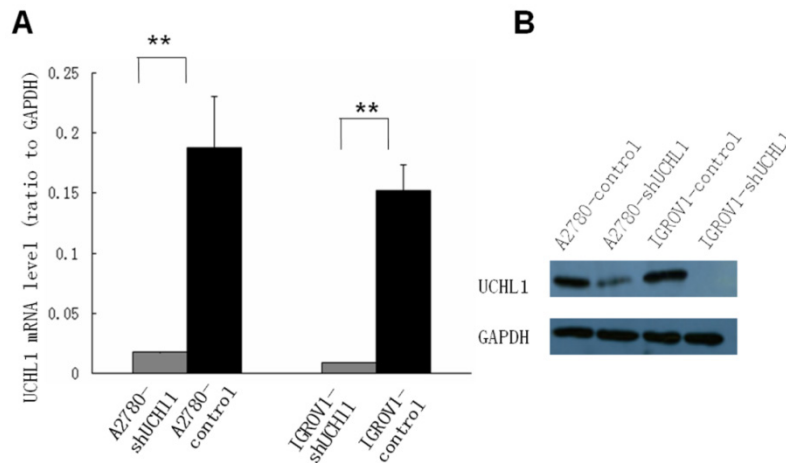
To study the function of UCHL1 in ovarian can-



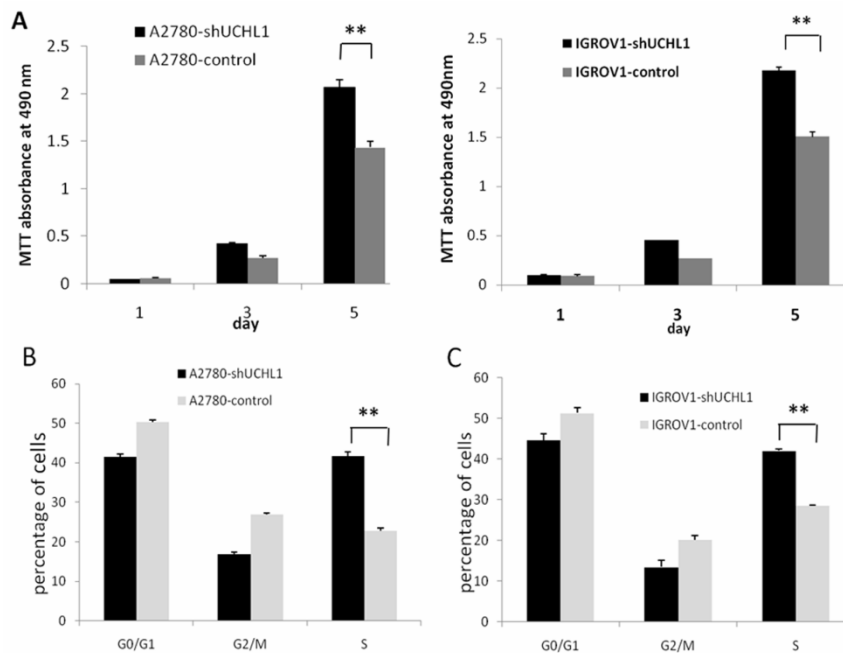
**Fig 1.** Silencing of UCHL1 was related to its promoter hypermethylation and UCHL1 expression could be restored by 5-Aza-dc treatment. (A) UCHL1 expression in seven ovarian cancer cell lines. (B) Bisulfite-modified DNA sequencing of UCHL1 promoter in seven ovarian cancer cell lines. Open circles, unmethylated; closed circles, methylated; yellow, unknown methylation status. (C) Methylation status of UCHL1 promoter in seven ovarian cancer cell lines. M, methylated; U, unmethylated. (D) Methylation status of UCHL1 in A2780CP cells treated with/without 10 μM 5-Aza-dc. M, methylated; U, unmethylated. (E) UCHL1 expression could be restored by 5-Aza-dc treatment in A2780CP cells.

cer, two ovarian cancer cell lines, A2780 and IGROV1, which express relatively high level UCHL1, were chosen to perform UCHL1 knockdown. Lentivirus infection led to a high and stable knockdown-efficiency in both A2780 and IGROV1 cells. UCHL1 expression was significantly reduced at mRNA level as well as protein level (Fig. 2).

We performed a cell growth curve assay to determine the effect of UCHL1-knockdown in A2780 and IGROV1 cells. As shown in Fig. 3A, MTT absorbance at 490 nm of A2780-shUCHL1 and A2780-control differed significantly on the 5<sup>th</sup> day, indicating that reduced expression of UCHL1 in A2780 cells increased cell proliferation. The same situation was detected between IGROV1-shUCHL1 and IGROV1-control. Furthermore, cell cycle analysis revealed up-regulation of S phase both in A2780-shUCHL1 and IGROV1-shUCHL1 (Fig. 3B-C), indicating that UCHL1 knockdown promoted cell proliferation by progressing cell cycle.



**Fig 2.** Suppression of UCHL1 expression by shRNA. Lentivirus harboring shRNA against UCHL1 or Non-Mammalian shRNA was used to infect UCHL1-positive A2780 and IGROV1 cell lines. Expression of UCHL1 was determined by quantitative real-time polymerase chain reaction (qRT-PCR) (A) and Western Blot (B). Both A2780-shUCHL1 and IGROV1-shUCHL1 showed a significant reduction of UCHL1 expression. \*\*,  $p < 0.01$ .



**Fig 3.** Effect of UCHL1 knockdown on cell proliferation and cell cycle. (A) Cell proliferation ability was determined by MTT assay. Data was shown by mean of spectrometric absorbance  $\pm$ SD of four independent experiments. (B) Cell cycle of A2780-shUCHL1 and A2780-control was analyzed by flow cytometry. (C) Cell cycle of IGROV1-shUCHL1 and IGROV1-control was analyzed by flow cytometry.

Then we assessed the effects of UCHL1 knockdown on apoptosis. We found that reduced UCHL1 expression resulted in reduction of apoptosis. In A2780-shUCHL1 cells, this effect was especially significant ( $p < 0.05$ ) (Fig. 4A). IGROV1-shUCHL1 cells also showed a similar trend while the effect was not statistically significant (Fig. 4B).

### 3.3 Reduced expression of UCHL1 in A2780 and IGROV1 induces cisplatin resistance

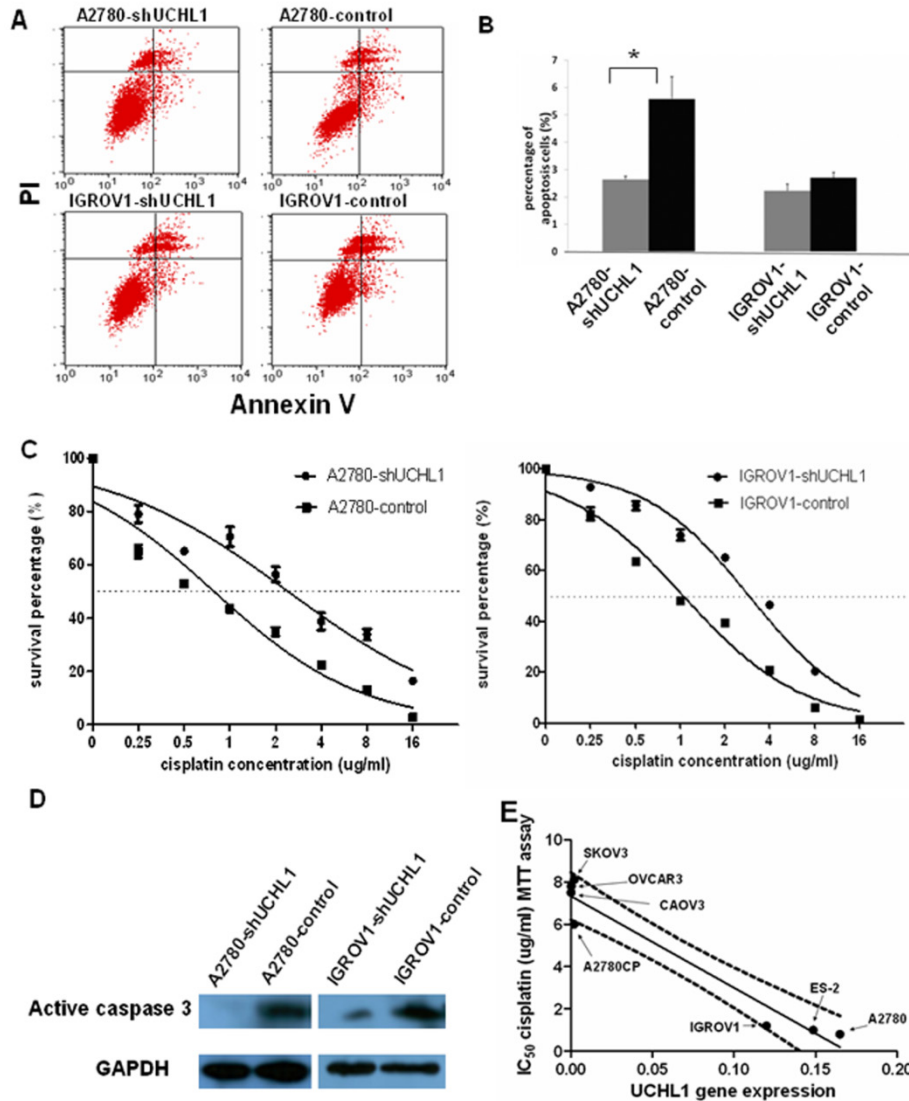
Most anti-cancer reagents work by triggering apoptosis in tumor cells and the ability to induce apoptosis determines the therapeutic efficacy. Since suppression UCHL1 reduced apoptosis, we per-

formed MTT assay to determine whether UCHL1 knockdown in ovarian cancer cells influences resistance to cisplatin, a common reagent used to treat ovarian cancer. As shown in Fig.4C, A2780-shUCHL1 and IGROV1-shUCHL1 showed relatively high cisplatin resistance compared to their controls, with  $IC_{50}$  increasing from 0.8 $\mu$ g/ml to 2.4 $\mu$ g/ml for A2780-control and A2780-shUCHL1, and from 1.2 $\mu$ g/ml to 3.2 $\mu$ g/ml for IGROV1-control and IGROV1-shUCHL1. Furthermore, we conducted western blot to determine cleaved caspase 3 expression after cisplatin treatment of these cells. As expected, there was a decrease in cleaved caspase 3 expression in UCHL1 knockdown cells (Fig.4D), indi-

cating that reduced expression of UCHL1 in A2780 and IGROV1 made the cells become resistant to cisplatin-induced apoptosis.

We questioned whether UCHL1 expression level correlates with cisplatin resistance in ovarian cancer. We measured UCHL1 mRNA expression level and determined cisplatin resistance with MTT assay in a panel of ovarian cancer cell lines. SKOV3, OVCAR3 are derived from metastatic tumors of patients treated with chemotherapy and are resistant to cisplatin.

CAOV3 also exhibited cisplatin resistance although it's sensitive to other chemotherapy agent<sup>21</sup>. A2780, ES-2, and IGROV1, derived from untreated patients, are sensitive to cisplatin<sup>22,23</sup>. A2780CP, a parental cell line of A2780, is resistant to cisplatin<sup>24</sup>. We found that UCHL1 expression levels were negatively correlated with IC<sub>50</sub> values from cisplatin MTT assay with a correlation coefficient of -0.96 (\*\*, p<0.001), which was statistically significant (Fig. 4E).



**Fig 4.** Knockdown of UCHL1 reduced cell apoptosis and contributed to cisplatin resistance. (A) Cell apoptosis ratio was analyzed by flow cytometry. Shown were representative apoptosis figures of each cell line and data was summarized in (B). (C) UCHL1 knockdown induced cisplatin resistance in ovarian cancer cell lines. A2780-shUCL1, A2780-control, IGROV1-shUCL1 and IGROV1-control were treated with cisplatin in different doses from 0µg/ml to 16µg/ml for 48 hours. Then MTT assay was performed to measure the cell survival. Dotted line, 50% survival. (D) Total protein was extracted from the four cell lines which were treated with 20µg/ml cisplatin for 16 hours and analyzed with active caspase 3 antibody, with GAPDH as internal control. (E) Expression of UCHL1 in diverse ovarian cancer lines negatively correlates with cisplatin resistance. UCHL1 mRNA expression and cisplatin resistance in a panel of 7 ovarian cancer cell lines were negatively correlated, with correlation coefficient -0.96 (p<0.001). Dotted line, 95% confidence interval.

### 3.4 Identification of biological process regulated by UCHL1 suppression

To explore the mechanisms of how reduced expression of UCHL1 promotes cell proliferation, reduces apoptosis and confers cisplatin resistance, we performed a microarray analysis to identify different genes regulated by UCHL1-knockdown. The array data was submitted to the GEO database (GSE43884) (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43884>). Comparing gene expression profile between A2780-shUCHL1 and A2780-control, we identified 1549 differentially expressed genes (724 down-regulated and 825 up-regulated) with fold change >2 (Supplementary Table 1). We noted up-regulation of several apoptosis related genes including apoptosis regulator BCL2 (B-cell CLL/lymphoma 2) (up 4.1 folds), BCL11A (B-cell CLL/lymphoma 11A) (up 29 folds), AEN (apoptosis enhancing nuclease) (up 2.0 folds) and XIAP (X-linked inhibitor of apoptosis) (up 2.6 folds).

We next focused on BCL2, an important regulator of apoptosis, for validation. First, we validated that BCL2 also was up-regulated at protein levels in knock-down cell lines A2780-shUCHL1 and

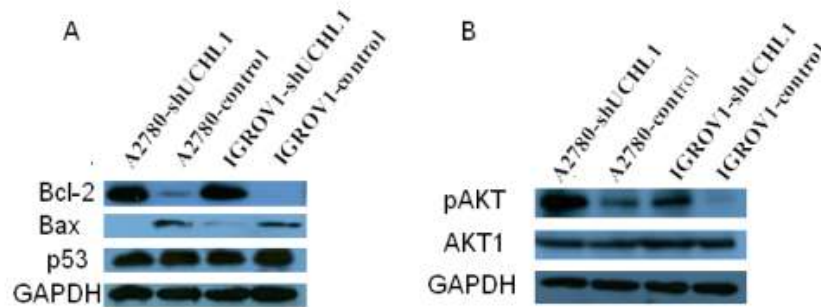
IGROV1-shUCHL1 (Fig. 5A). Another member of BCL2 family Bax, which heterodimerizes with Bcl-2 to accelerate cell apoptosis<sup>25</sup>, was down-regulated (Fig. 5A). As p53 expression was reported to be altered in UCHL1 over-expression or knockdown cells<sup>9,11,26</sup>, we also determined p53 level using western blot. However, we did not observe any significant alteration of its expression (Fig. 5A). Moreover, we detected higher pAKT expression in A2780-shUCHL1 and IGROV1-shUCHL1 while no significant alteration of AKT1 was detected, suggesting that the AKT pathway, rather than p53 signaling pathway might be involved in UCHL1 regulated apoptosis in these ovarian cancer cells.

Gene ontology analysis by GoMiner of these differentially expressed genes, with all gene list as total file, showed that 24 GO terms were found to be enriched, including positive regulation of apoptosis (GO:0043065), regulation of cell death (GO:0010942), negative regulation of apoptosis (GO:0043066), which were top three enriched GO terms (Table 1). This result was consistent with our observations, strongly indicating that UCHL1 is likely to be involved in the regulation of apoptosis.

**Table 1.** Enriched GO terms in biological process for UCHL1 regulated genes.

GO Category	Total Genes	Changed Genes	Enrichment	LOG10(p)	False Discovery Rate
GO:0043065 positive regulation of apoptosis	488	59	1.47	-2.77	0.05
GO:0010942 positive regulation of cell death	496	62	1.52	-3.25	0.03
GO:0043066 negative regulation of apoptosis	416	52	1.52	-2.82	0.05
GO:0030097 hemopoiesis	335	46	1.67	-3.41	0.03
GO:0048534 hemopoietic or lymphoid organ development	360	50	1.69	-3.78	0.02
GO:0002520 immune system development	388	51	1.60	-3.26	0.03
GO:0007507 heart development	245	35	1.74	-3.04	0.05
GO:0060537 muscle tissue development	180	28	1.89	-3.11	0.04
GO:0014706 striated muscle tissue development	171	28	1.99	-3.48	0.03
GO:0001934 positive regulation of protein phosphorylation	130	23	2.15	-3.45	0.03
GO:0001932 regulation of protein phosphorylation	235	37	1.91	-4.03	0.01
GO:0051493 regulation of cytoskeleton organization	153	28	2.22	-4.34	0.01
GO:0045165 cell fate commitment	144	24	2.03	-3.18	0.04
GO:0045185 maintenance of protein location	75	15	2.43	-3.00	0.04
GO:0042594 response to starvation	63	14	2.70	-3.31	0.03
GO:0009267 cellular_response_to_starvation	49	13	3.23	-3.93	0.01
GO:0045580 regulation of T cell differentiation	54	12	2.70	-2.92	0.05
GO:0010332 response to gamma radiation	29	10	4.19	-4.19	0.01
GO:0048538 thymus development	25	10	4.86	-4.85	0.01
GO:0030856 regulation of epithelial cell differentiation	33	10	3.68	-3.66	0.03
GO:0043588 skin development	33	9	3.32	-2.98	0.05
GO:0048730 epidermis morphogenesis	27	8	3.60	-2.96	0.05
GO:0030035 microspike assembly	31	9	3.53	-3.19	0.04
GO:0031998 regulation of fatty acid beta-oxidation	12	6	6.08	-3.74	0.02





**Fig 5.** Detection of apoptosis related proteins by western blot. (A) Up-regulation of Bcl-2/Bax but no significant alteration of p53 were detected in UCHL1 knockdown cells. (B) Up-regulation of AKT pathway activity was found in UCHL1 knockdown cells.

#### 4. Discussion

In this study, we described, for the first time, how UCHL1 modulates cisplatin resistance in ovarian cancers. We showed that knockdown of UCHL1 in ovarian cancer cells promoted cell proliferation (Fig. 3A) by inducing S phase cell cycle (Fig. 3B-C) and reduced apoptosis (Fig. 4A-B). Caspase 3, a frequently activated apoptosis protease and a typical hallmark of apoptosis<sup>27</sup>, is reduced upon UCHL1 knockdown (Fig. 4D). Our data is consistent with previous reports of UCHL1 in other types of cancers. UCHL1 over-expression has been reported to induce cell growth arrest in prostate cancer<sup>15</sup> and breast cancer<sup>26, 28</sup>. In breast cancer, ectopic expression of UCHL1 is able to induce apoptosis by stabilizing p53. In nasopharyngeal carcinoma, UCHL1 was found to form a complex with p53/MDM2/ARF to promote p53 signaling<sup>9</sup>. In hepatocellular carcinoma, it was reported that UCHL1 serves as a tumor suppressor gene by directly interacting with p53 and stabilizing p53<sup>11</sup>. Yet in our study, no significant alteration of p53 was found (Fig. 5A), suggesting UCHL1 mediated apoptosis in other ways rather than by regulating p53.

We initially observed that UCHL1 expression is anti-correlated with cisplatin resistant levels (as measured by IC<sub>50</sub>) (Fig. 4E). This is consistent with a UCHL1 expression at the GEO database showing that UCHL1 was reduced to no expression in the tissues from carboplatin resistant ovarian cancer patients ([http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS1381:36990\\_at](http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS1381:36990_at)) compared to the carboplatin sensitive tissues (Supplementary Fig. 1)<sup>29</sup>. Carboplatin, a parental platinum compound of cisplatin, shares similar biochemical characters as well as similar anti-cancer mechanisms with cisplatin<sup>30</sup>. The fact that cross resistance between carboplatin and cisplatin<sup>31, 32</sup> ensure us making use of the GEO dataset.

We further observed that methylation of the UCHL1 promoter correlated negatively to the expres-

sion of UCHL1 (Fig. 1A-C). Demethylation of A2780CP with Aza restored UCHL1 expression, confirming promoter methylation was responsible for its silencing (Fig. 1D-E). This is consistent with the previous observation by Okochi-Takada et al. who showed that UCHL1 was silenced in about half of the ovarian cancer cell lines that they tested and the expression was also correlated with promoter methylation status<sup>33</sup>. Moreover, we have extended this finding to further experimentation and showed that knockdown of UCHL1 increased cisplatin resistance in both A2780 and IGROV1 ovarian cancer cells (Fig. 4C). Our data demonstrated, for the first time, that UCHL1 expression directly regulated cisplatin resistance in ovarian cancers.

We also performed microarray expression profiling to understand the global picture of UCHL1's effects on ovarian cancer cells. GoMiner analysis showed that apoptosis and cell death related biological processes are enriched in UCHL1 regulated genes. In particular, we found that several apoptosis related genes including apoptosis regulator BCL2 (B-cell CLL/lymphoma 2), BCL11A (B-cell CLL/lymphoma 11A), AEN (apoptosis enhancing nuclease) and XIAP (X-linked inhibitor of apoptosis). AEN was recently identified as a direct target gene of p53<sup>34</sup>. Bcl-2/Bax ratio is a determinant of cell fate and was considered to be a "rheostat" that regulates apoptosis<sup>35, 36</sup>. We found up-regulated Bcl-2/Bax ratio in UCHL1-knockdown cells (Fig. 5A), however, no significant alteration of p53 was found (Fig. 5A).

AEN induces apoptosis by itself, and more importantly, it is also required for efficient DNA fragmentation in p53-dependent apoptosis<sup>34</sup>. The role of AEN in UCHL1 knockdown induced apoptosis remains to be studied. XIAP regulates AKT activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells<sup>37</sup>. Interestingly, in our study we found up-regulated AKT activity in UCHL1-knockdown



ovarian cancer cells (Fig. 5B). Downregulation of XIAP inhibits proliferation, induces apoptosis, and reverses the cisplatin resistance of ovarian carcinoma<sup>38</sup>. Here we demonstrated that UCHL1 regulated the expression of AEN and XIAP, suggesting that UCHL1 might be a key master regulator of cisplatin resistance in ovarian cancers. Further experimentation is warranted.

In summary, we present data showing that UCHL1 might be a tumor suppressor in ovarian cancers. Furthermore, UCHL1 expression level is negatively related to cisplatin resistance in ovarian cancer and knockdown of UCHL1 increased cisplatin resistance. GoMiner analysis showed that apoptosis and cell death related biological processes are enriched in UCHL1 regulated genes. Our study provides a foundation to develop promising strategy to overcome cisplatin resistant ovarian cancer via UCHL1 mediated pathways.

## Supplementary Material

Supplementary Table 1

[<http://www.jcancer.org/v04p0662s1.pdf>]

Supplementary Figure 1

[<http://www.jcancer.org/v04p0662s2.pdf>]

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## Competing Interests

Biaoyang Lin received research funding from Hangzhou Proprium Biotech Co. The other authors have declared that no competing interest exists.

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