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Upregulation of Ubiquitin Carboxyl-Terminal Hydrolase L1 (UCHL1) Mediates the Reversal Effect of Verapamil on Chemo-Resistance to Adriamycin of Hepatocellular Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1,2,3 Guangshan Yang*
BCDE 2 Gaofei Fan*
ABCDEF 2 Tengyue Zhang*
ABCD 4 Kelong Ma*
BC 2 Jin Huang
AB 2 Miao Liu
AB 2 Xiaolu Teng
AB 2 Kun Xu
ACDFG 1,2 Pingsheng Fan
ACDFG 5 Dongmiao Cheng

1 School of Clinical Medicine, Shan Dong University, Jinan, Shandong, P.R. China
2 The Cancer Hospital of Anhui Province, Hefei, Anhui, P.R. China
3 Anhui Provincial Hospital, Hefei, Anhui, P.R. China
4 Clinical College of Integrated Traditional Chinese and Western Medicine, Anhui University of Chinese Medicine, Hefei, Anhui, P.R. China
5 Department of Radiotherapy, The First People's Hospital of Huainan City, Huainan, Anhui, P.R. China

* These authors have contributed equally to this work

Corresponding Author: Pingsheng Fan, e-mail: fanpingsheng@csco.org.cn; Dongmiao Cheng, e-mail: 18949669862@163.com

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Background: The aim of this study was to investigate the role of ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) in the reversal effect of verapamil (VER) on chemo-resistance to Adriamycin (ADM) in treatment of hepatocellular carcinoma (HCC).

Material/Methods: HCC cell lines SMMC-7721 and BEL-7402 were used as model cell lines. High-throughput transcriptome sequencing based on Illumina technology was used to screen whether UCHL1 mediated the reversal effect of VER on chemo-resistance. Quantitative real-time PCR (qRT-PCR) was performed to determine the expression level of UCHL1 mRNA in HCC cells, and western blot analysis was performed to examine the protein expression of UCHL1 protein in HCC cells. Immunohistochemistry assay was performed to determine the protein expression of UCHL1 in tissue samples from patients presenting with either positive or negative responses to the reversal therapeutic regimen of VER. Moreover, cell models with UCHL1 knockdown and overexpression were established to examine the reversal effect of VER on chemo-resistance to ADM in HCC cells. Cell apoptosis was determined by flow cytometry following Annexin V-PI staining.

Results: The expression levels of UCHL1 genes correlated with the level of apoptosis induced by ADM+VER. Overexpression of UCHL1 genes promoted apoptosis in cells treated with VER+ADM. UCHL1 knockdown using siRNA weakened the effect of ADM+VER, indicating that ADM+VER promotes HCC cell apoptosis and that UCHL1 genes participate in VER-mediated promotion in tumor cell apoptosis.

Conclusions: Upregulation of UCHL1 enhanced the reversal effect of VER on chemo-resistance to ADM and promoted cell apoptosis. The underlying mechanism of the function of UCHL1 and the signaling pathway involved in its effect are to be investigated in our future research.

MeSH Keywords: Antineoplastic Combined Chemotherapy Protocols • Carcinoma, Hepatocellular • Drug Resistance • Epirubicin • Ubiquitin Thiolesterase • Verapamil

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Background

Hepatocellular carcinoma (HCC) is a common solid tumor, which ranks the second in mortality among all human malignancies and presents as a serious public health issue [1]. Due to the lack of early warning signs and rapid progression, the majority of patients are diagnosed at a late stage and miss the optimal window for operative treatment. Currently, transcatheter arterial chemoembolization (TACE) is considered an important therapeutic regimen for HCC patients in an advanced disease stage. However, TACE is contraindicated when portal vein thrombosis and extrahepatic tumor metastasis occur [2]. Chemotherapeutics and target therapies have become the main measures used to prolong the survival of patients with HCC, although the therapeutic efficacy can be compromised by chemo-resistance of the tumor. Analysis of multiple randomized clinical trials showed that response rates to the current standardized treatment regimen was only about 50% and the most effective target therapeutic agent, sorafenib, only prolonged the survival of patients for 3 months [3,4]. Therefore, investigating the approach to overcome resistance to chemotherapeutic is imperative to improve the clinical outcomes for HCC patients. Verapamil (VER) has been found to reverse chemo-resistance in a variety of human cancer cells [5]. *In vitro* studies showed that the effective dosage of VER to reverse chemo-resistance ranged from 6.0 μmol/L to 10.0 μmol/L. However, the safety concentration of VER was only 1.0 to 2.0 μmoL/L. Above this range, VER treatment could result in serious adverse effects such as sinus bradycardia and atrioventricular block [6], which limits its use in reversal of chemo-resistance. In our clinic practice, we combined VER with TACE treatment and found that it significantly improves the clinical outcomes of HCC patients [7]. With this treatment regimen, the overall effective rate reached 71.4% and the 1-year survival rate was increased to 81.80%, which surpassed the therapeutic efficacy of the standardized treatment regimen [8]. However, about 30% of patients did not showed good response to our treatment regimen, which may be attributed to the differential capability of VER in reversal of chemo-resistance.

In our previous study, we tested the reversal effect of VER on chemo-resistance to oxaliplatin (L-OHP), Adriamycin (ADM), and 5-fluorouracil (5-FU) in 4 HCC cell lines (SMMC-7721, BEL-7402, HepG2, and QGY-7703) to screen a number of target genes that may mediate the reversal effect of VER on chemo-resistance. Among these genes, ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) may be one of the candidate gene [9]. In this study, we conducted experiments to verify the role of UCHL1 in the reversal effect of VER on chemo-resistance.

Material and Methods

Experimental materials

Through our hospital pharmacy, we obtained VER from Shanghai Hefeng Pharmaceutical Company at 5 mg/2 mL, oxaliplatin (L-OHP) from Jiangsu Hengrui at 50 mg/ampul, doxorubicin hydrochloride (ADM) from Zhejiang Haizheng at 10 mg/ampul, 5-FU from Jiangsu Nantong Jinghua Pharmaceutical Company at 0.25 g/10 mL. Cell Counting Kit 8 (CCK-8) was provided by Japanese colleagues at the Chemical Institute. The Tiangen Company provided RNA extraction and reverse transcription kit and 2×SYBR Green Universal qPCR Master Mix. Mouse anti-human UCHL1 primary antibody was obtained from the Abcam company (USA). GAPDH antibody was purchased from Sigma; goat anti-mouse HRP-labeled secondary antibody was obtained from Guizhou Jinqiao Biological Company; and high-throughput sequencing was commissioned by Guangzhou Ruibo company. The Guangzhou Ruibo Company also provided siRNA for gene transfection; empty vector, and overexpression plasmid was purchased from the Origene Company, TrueORF GOLD model. The Beijing Beibo Company provided Annexin V-PI double staining kit; Lipofectamine 3000 was obtained from Invitrogen Company; Shanghai Shanjing Biotechnology Company conducted primer design and synthesis.

Cell culture

High glucose DMEM medium supplemented with 10% FCS was applied to culture human hepatoma cell line cells at 37°C, 5% CO_2, and saturated humidity. The cells were treated with 0.25% trypsin and cultured to logarithmic growth phase. When the cells had grown to distribution of monolayers, they were washed with PBS, and 0.25% trypsin was used to digest cells to passage as 1: 3, at the logarithmic growth phase, the cells were tested.

High throughput transcriptome sequencing based on Illumina sequencing platform

In this study, 2 HCC cell lines (SMMC-7721 and BEL-7402) that had the most significant differences in resistance to ADM chemotherapeutic drug reversed by VER in our previous study were sequenced by high-throughput sequencing. Cells were grouped into a normal (NC) group, a VER alone (VER) group, tan ADM alone (ADM) group, and a VER combined with ADM (ADM + VER) group. Taking the toxicities of chemotherapeutic drugs and follow-up tests into consideration, the dose of ADM was chosen as 1/5 times the concentration of $IC50_1$ in the cell line, the dose of ADM was 1.14 ug/mL ($IC50_1$ value: 5.71 ug/mL) in SMMC-7721 cells, 2.28 μg/mL ($IC50_1$ value: 11.40 μg/mL) in BEL-7402 cells, the dose of VER was 4.91 ug/mL.

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Using Hiseq genome-wide sequencing, the bioinformatics analysis based on alignment with reference genomes was carried out to screen out the genes that expressed differently between the 2 cell lines before and after using ADM and ADM+VER.

Real-time quantitative PCR detecting the expression of UCHL1 in hepatoma cells

Primer design

Primer 5.0 software was used to design primer:
hUCHL1F: 5'-CCGAGATGCTGAACAAAG-3',
hUCHL1R: 5'-CAGAGACTCCTCTTCCAG-3';
internal primer (GAPDH):
F: 5'-ATCTCTGCCCCCCTGCTGA-3',
R: 5'-GATGACCTTGCCCACAGCCT-3'

RNA extraction and detection of the target gene

RNA extraction and quantification were carried out according to kit instructions, cells were grouped following the 1.3 transcriptome sequencing grouping. First, annealing temperature and primer specificity were optimized after primers were diluted. The reaction mixture was then prepared in accordance with the following reaction system: 12.5 μL of 2×SYBR Green universal qPCR Master Mix, 1.5 μL of each upstream/downstream primer, 3 μL cDNA, and using double distilled water added to a final volume of 25 μL. The appropriate volume of reaction mixture was prepared according to the number of test samples, and correspondingly added to the PCR plate, adding 25 μL to each hole. The reaction mixture was centrifuged to the bottom of the tube. The PCR was conducted according to the following reaction conditions: pre-denaturation at 95°C for 15 min, PCR reaction (denaturation at 95°C for 10 secs, annealing/extension at 60°C for 32 secs for a total of 40 cycles), and to build a dissolution curve, the data was acquired expediently from real-time fluorescence quantitative PCR instrument.

Western blot detecting the expression of UCHL1 in HCC cells

Cells were seeded on a 6-cm plate (5.0×10^5 cells per well) with groups of 1.3 transcriptome sequencing. After drug treatment, cells were harvested and lysed with RIPA lysate (supplemented with protease inhibitor PMSF) for 30 min. After centrifugation at low temperature, the supernatant was taken, and bovine serum albumin was applied as a standard to quantify protein. The protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes. After blocking with 5% non-fat dry milk for 1 hour, primary antibody (β-actin 1: 2000, UCHL1: 1000) was added to incubate overnight at 4°C. PVDF membranes transferred with protein were washed with phosphate buffered saline (PBST) 4 times (10 min each time), incubated with secondary antibody (1: 5000) for 2 hours, and washed with PBST. Signals were monitored using a chemiluminescent substrate.

Immunohistochemistry detecting the expression of UCHL1 protein in HCC tissue samples

Clinical data and grouping

Twenty-two patients with medium and advanced primary HCC, who were admitted to our hospital were enrolled in this study. All specimens were obtained by liver puncture technique. The pathology was confirmed as primary HCC. Each patient received a total of 2 to 4 interventions per month. To evaluate patient treatment efficacy, patients were divided into 2 groups: effective VER anti-resistance treatment group (treatment significantly improved cases, CR) in 12 cases, and ineffective VER anti-resistance treatment group (treatment of disease cases, PD) in 10 cases. Comparison of gender and age had no significant differences between the 2 groups of patients who signed informed consent to participate in the group of VER combined with TACE treatment.

VER combined with TACE treatment and efficacy evaluation criteria

Seldinger technique was used to puncture the femoral artery, celiac artery, or variant target vessel to infiltrate VER, doxorubicin (ADM), oxaliplatin (L-OHP), and 5-FU, and then selectively lead the catheter into the variant target vessel or the right or left hepatic artery, iodized oil emulsion embolism. Drug infusion step was: VER 25 mg, ADM 40~50 mg/m^2, L-OHP 100~150 mg/m^2, 5-FU 1000 mg/m^2. According to the results of angiography, we selected the type of embolization material and estimated the amount of embolization agent; after embolization, arterial angiography was applied to understand the situation of hepatic artery occlusion, extubation after embolization satisfied, and compress puncture point for 10~20 min to stop bleeding. After observing no bleeding, local pressure bandaging was applied.

The evaluation of curative effect of the primary liver cancer included: 1) clinical cure or significant improvement (CR) defined as tumor disappeared or reduced more than 75%, lipiodol uniform accumulation, tumor blood vessels were completely occluded or tumor margin only remains a small number of tumor blood vessels or tumor staining; 2) improvement (PR) defined as the mass of the tumor narrowed by about 30–75%, uneven accumulation of lipiodol, and the area of the lipiodol filled up was more than 1/2 of the tumor area. The tumor blood vessels decreased significantly was 3) stability (SD) defined as the mass of the tumor narrowed by less than 30%, lipiodol was spotted flocculent accumulation, lipiodol filling area was

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less than 1/2 tumor, no significant reduction in tumor blood vessels; and 4) progressive or worsening (PD) was defined as: lumps were increased, iodized oil was scattered spotted accumulation or no significant accumulation of iodized oil, the area of lipiodol accumulation was less than 1/3 tumor area, tumor vessels increased significantly to form a new hepatic artery portal vein fistula or hepatic artery hepatic vein fistula.

Immunohistochemical methods and results analysis

Liver cancer paraffin embedded tissue sample was taken: 4-μm serial sections and processed with dewaxing hydration. Boiling 15 min using microwave repair antigen, the first antibody was diluted 1: 100. Immunohistochemistry was as follows: using sodium citrate buffer hot repair antigen, conventional SP immunohistochemical staining, DAB staining, hematoxylin counterstain. Known liver cancer positive sections served as a positive control, and PBS replaced the first antibody as a negative control.

Positive cells were expressed as brown granules on the cell membrane and/or cytoplasm. The semi-quantitative results were used to determine the average optical density (IOD/area, density mean) of the positive expression area using Image-Pro Plus 6.0 (IPP). The mean and standard deviation of each photograph of the same experimental group were calculated, and statistically analyzed as to whether there was a significant difference between the average optical density values of each experimental group.

Liver cancer cells UCHL1 gene-specific transfection experiments

Plasmid

UCHL1 gene overexpression plasmid (PCMV6-AC-GFP) and empty vector (PCMV6-Entry) were purchased from Origene Company TrueORF GOLD, specific siRNA interference plasmids was designed according to the siRNA sequence (siR-UCHL1) for this gene using software provided by Reebok Company. The specific siRNA sequence for UCHL1 was constructed: CCGGGTGTGAGCTTCAGATGGTGAACTCGAGTTCACCATCTGAAGCTC ACACTTTTT.

Cell transfection

The cell transfection used the following steps: 1) HCC cells were cultured in high-glucose DMEM medium supplemented with 10% fetal bovine serum (FBS) with antibiotics at 37°C in 5% $C0_2$ saturated humidity incubator to logarithmic growth phase. 2) one day before transfection, cells in the logarithmic growth phase were digested with 0.25% trypsin, resuspended in high glucose DMEM medium containing 10% FBS without antibiotics and inoculated into 6-well plate (5.0×10^5 cells) or 96-well plate (1.0×10^4 cells), and the transfection was started when grown reached 80–90% confluence. 3) Cells were transfected following the Lipofectamine 3000 transfection method: 5 uL (6-well plate) and 0.25 uL (96-well plate) of Lipofectamine 3000 were respectively added to 125 uL (6-well plate) and 6.25 uL (96-well plate) Optim-MEM medium, gently mixed and incubated for 5 min at room temperature. 4) Plasmid was added as 0 uL (6-well plate) or 0.5 uL (96-well plate) to 125 uL (6-well plate) or 6.25 uL (96-well plate) Optim-MEM culture medium, gently mixed and incubated for 5 min at room temperature. 5) the mixture from step 3 was slowly added dropwise into cells from step 4 and gently mixed and incubated for 20 min at room temperature. 6) the mixture of step 5 was slowly added to the cell culture dish, drop by drop, being careful not to rinse the cells. For step 7), cells were cultured in 37°C, 5% CO_2 saturated humidity incubator, then 5 hours later, replaced with double antibody-free DMEM culture medium containing serum, and analyzed after cultured for 72 hours.

Annexin V-PI double staining was applied to detect the apoptosis

The cells were grouped based on reference to the transcriptome sequencing, and the cells were cultured in a 6-well plate with RMPI-1640 containing 10% FBS for 24 hours until confluency was about 70%. After trypsinization, which was terminated by medium supplemented with 10% FBS, the cells were centrifuged at 1000 rpm for 5 min at 4°C and washed twice with pre-cooled PBS. Cells were resuspended in 100 μL of binding buffer and then 2 μL of Annexin V-FITC was added. Cells were mixed well, kept in the dark for 15 min on ice, then 400 uL of PBS and 1 uL of PI solution were added to each sample before detection using flow cytometer; the apoptosis rate of hepatoma cells was detected, and the status of apoptotic cells was observed using fluorescence microscope.

Statistical methods

Using the Illumina Hiseq 2000 sequencing platform, clean reads were aligned to human reference gene set (version hg38) using the Bowtie2 software. RSEM software was applied to quantified gene expression. DESeq (verison 1.28.0) software was used to perform differential gene analysis, the genes expressed differentially between samples were chosen from 2 levels of (|log 2 (fold change)|>1) and significant levels (q-value <0.05) [10,11]. The comparison of VER reversal drug resistance differences, the expression level of target genes, the level of apoptosis, and other continuous variables were described as mean ± standard deviation (x±S) for statistical description, One way ANOVA or Mann-Whitney U test was performed using Excel (Microsoft, Redmond, WA, USA) or Prism (Prism 4.0, GraphPad Inc, La Jolla, CA, USA) for data analysis. Experiments

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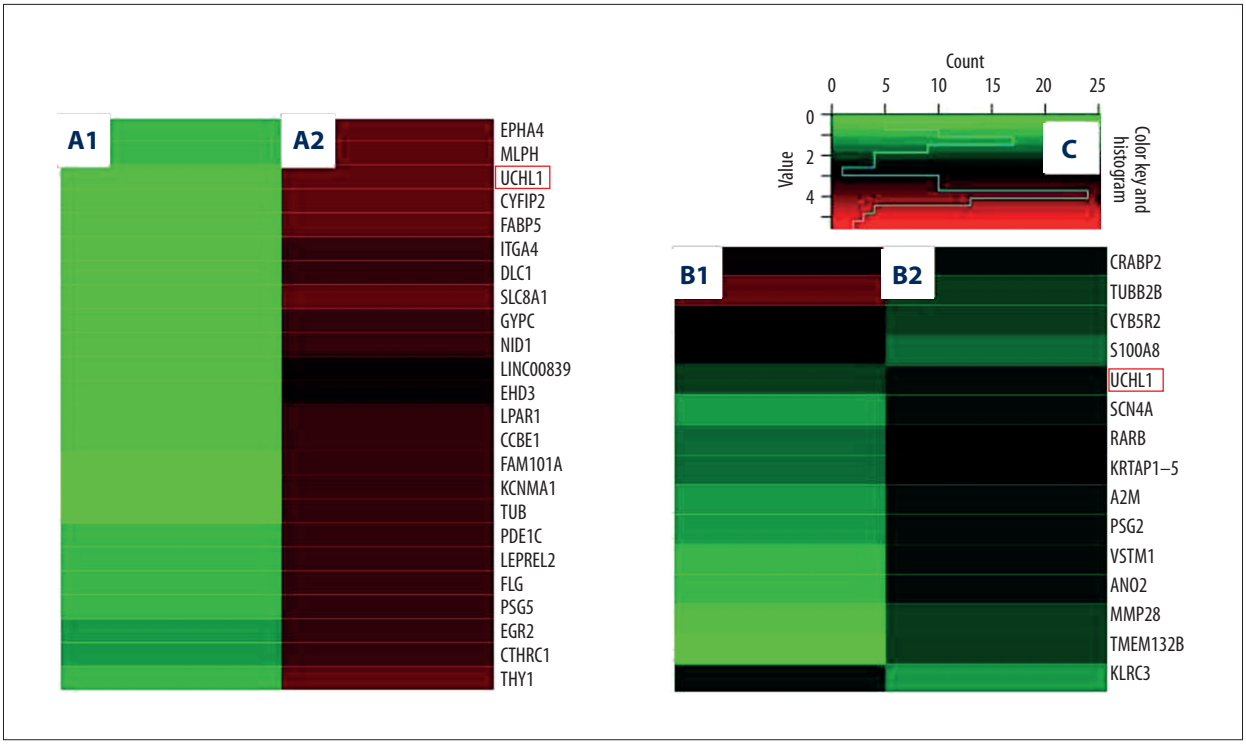

Figure 1. High-throughput transcriptome sequencing identifies differential genes which associated to the reversal effect of verapamil (VER) on chemo-resistance to Adriamycin (ADM) in BEL-7402 and SMMC-7721 cells. (**A1**) BEL-7402 ADM, (**A2**) BEL-7402 ADM+VER, (**B1**) SMMC-7721 ADM, (**B2**) SMMC-7721 ADM+VER.

were repeated more than 3 times, $P<0.05$ was considered as statistically significant.

Results

High-throughput transcriptome sequencing screens differential genes in HCC cells

It has been reported that the reversal effect of VER on chemo-resistance to ADM in BEL-7402 cells was significantly better than in SMMC-7721 cells. However, the mechanism which led to the difference remains to be elucidated [9]. Hence, in our present study, HCC cell lines SMMC-7721 and BEL-7402 were used as model cell lines, and the high-throughput transcriptome sequencing based on Illumina technology was used to sequence the potentially critical genes which mediated the reversal effect of VER on chemo-resistance in HCC. As shown in Figure 1, UCHL1 did not show significant differences between SMMC-7721 ADM and SMMC-7721 ADM+VER. However, UCHL1 was significantly elevated in both the BEL-7402 ADM group and the BEL-7402 ADM+VER group ($P<0.01$). Therefore, UCHL1 was used for future study.

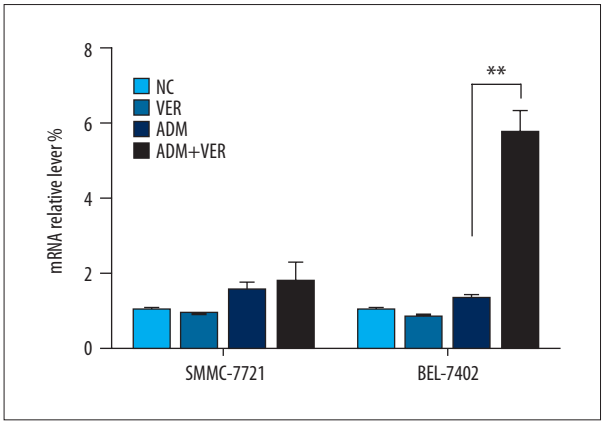

Figure 2. Quantitative real-time PCR (qRT-PCR) validates the expression of UCHL1 in SMMC-7721 and BEL-7402 cells. ** $P<0.01$. UCHL1 – ubiquitin carboxyl-terminal hydrolase L1.

qRT-PCR detects the expression of UCHL1 in HCC cells

According to aforementioned results, we further validate whether UCHL1 is the crucial gene which mediates ADM resistance. We applied qRT-PCR to detect the expression of mRNA UCHL1 in HCC cells. As shown in Figure 2, the expression of mRNA UCHL1 did not show significant differences between the SMMC-7721ADM group and the SMMC-7721ADM+VER

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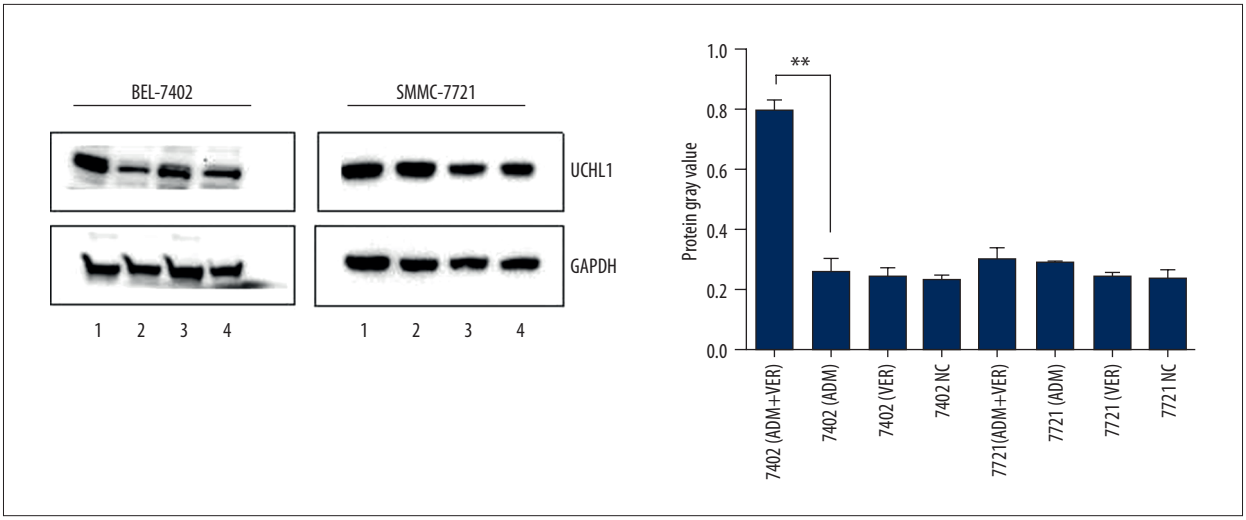

Figure 3. Western blotting detects the protein expression of UCHL1 in SMMC-7721 and BEL-7402 cells. ** *P*<0.01. UCHL1 – ubiquitin carboxyl-terminal hydrolase L1.

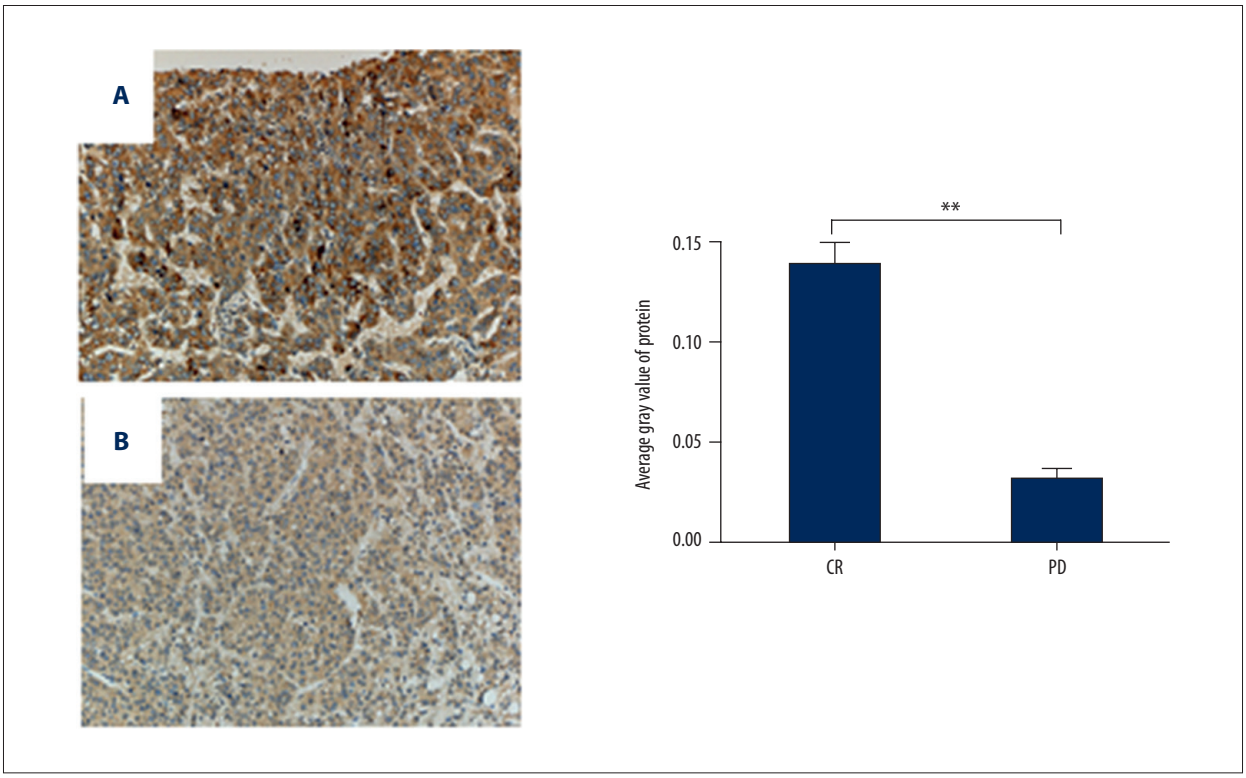

Figure 4. Representative images of UCHL1 expression in the tissue sample from patients presenting with a positive or a negative response to the reversal therapeutic regimen of verapamil. ** *P*<0.01. (**A**) Positive response, CR, 200×; (**B**) Negative response, PD, 200×). UCHL1 – ubiquitin carboxyl-terminal hydrolase L1.

group, while the mRNA and protein level of UCHL1 was significantly upregulated in both the BEL-7402 ADM group and the BEL-7402 ADM+VER group (*P*<0.01). These results suggest that UCHL1 might be a gene that mediates the reversal effect of VER on chemo-resistance to ADM in HCC.

Western blotting of the protein expression of UCHL1 in HCC cells

Next, we measured the expression of UCHL1 protein in SMMC-7721 cells and BEL-7402 cells. We also detected the expression of UCHL1 protein to further confirm its role in mediating the

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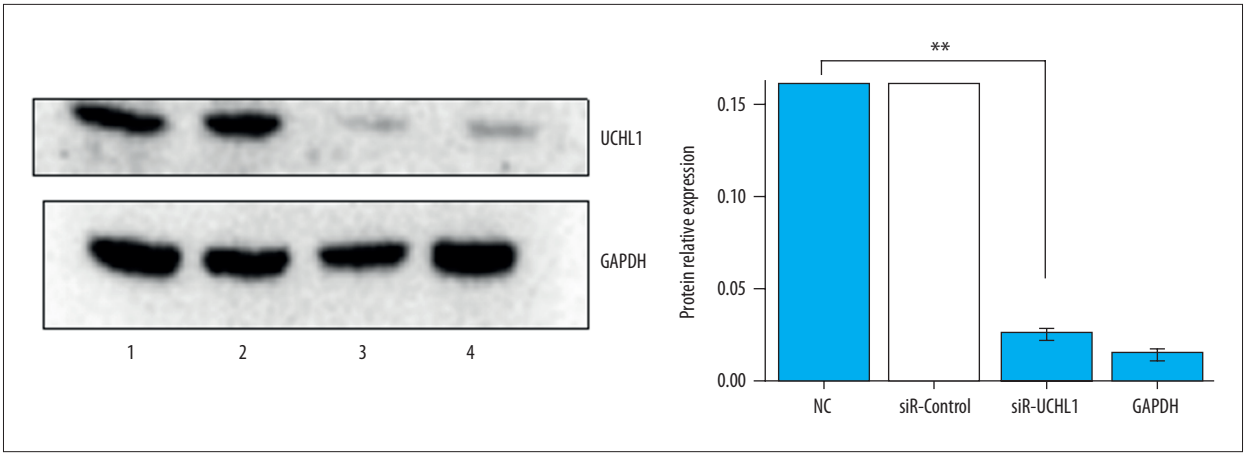

Figure 5. Transfection BEL-7402 cells with UCHL1 targeting siRNA, control siRNA, and western blotting detects the expression of UCHL1. UCHL1 – ubiquitin carboxyl-terminal hydrolase L1.

reversal effect of VER on chemo-resistance to ADM in HCC cells. As shown in Figure 3, the protein expression of UCHL1 did not show significant differences between the SMMC-7721 ADM group and the SMMC-7721 ADM+VER group, while UCHL1 was significantly upregulated in both the BEL-7402 ADM group and the BEL-7402 ADM+VER group ($P<0.01$). Collectively, we postulated UCHL1 might be a potential gene that mediates the reversal effect of VER on chemo-resistance to ADM in HCC cells.

Immunohistochemistry assay was performed in the protein expression of UCHL1 in the tissue samples from patients presenting either positive or negative response to the reversal therapeutic regimen of VER

Next, we also validated the expression of UCHL1 in the tissue samples from patients presenting with positive (12 cases) or negative (10 cases) response to the reversal therapeutic regimen of VER by using immunohistochemistry assay. As shown in Figure 4, UCHL1 was mainly distributed in the nucleus and cytoplasm. Furthermore, the expression of UCHL1 in the positive response group was significantly higher, when compared with the negative response group (** $P<0.01$).

Knockdown of UCHL1 significantly dampened the reversal effect of VER on chemo-resistance of BEL-7402 cells

We further tested to confirm whether UCHL1 was the critical gene that contributed to the reversal effect of VER on chemo-resistance in HCC cells. UCHL1 targeting siRNA or overexpression vector were used to manipulate the expression of UCHL1. After transfection, western blotting was used to measure the transfection efficiency. As shown in Figure 5, UCHL1 targeting siRNA effectively silenced the expression of UCHL1 in BEL-7402 cells. Next, after transfection, CCK-8 assay was applied to detect the IC50 in the BEL-7402 ADM group and the BEL-7402 ADM+VER group. As shown in Figure 6, knockdown of UCHL1 significantly dampened the reversal effect of VER on chemo-resistance of BEL-7402 cells. Moreover, SMMC-7721 cells were transfected with UCHL1 overexpression vector; Figure 7 shows transfection of UCHL1 overexpression vector effectively elevated the expression of UCHL1 in SMMC-7721 cells. We also detected the change of IC50 in the SMMC-7721 ADM group and the SMMC-7721 ADM+VER group by CCK-8 assay. As shown in Figure 6, overexpression of UCHL1 significantly enhanced the reversal effect of VER on chemo-resistance of in SMMC-7721 cells.

UCHL1 enhances the pro-apoptotic effect of VER in ADM resistant HCC cells

We also detected cell apoptosis in HCC cells. As shown in Figure 8, the apoptotic rate was 17.2% and 18.6% in the SMMC-7721 ADM group and the SMMC-7721 ADM+VER group, respectively. Data did not show a significant difference between the SMMC-7721 ADM group and the SMMC-7721 ADM+VER group. However, UCHL1 overexpression effectively enhanced the pro-apoptotic effect of VER in ADM resistant SMMC-7721 cells, the apoptotic rate was 16.3% and 22.5% in SMMC-7721 ADM and SMMC-7721 ADM+VER cells, respectively. In BEL-7402 ADM and BEL-7402 ADM+VER cells, the apoptotic rate was 14.9% and 23.6% respectively, suggesting VER could effectively enhanced the cell apoptosis in ADM resistance cells. After knockdown UCHL1 with siRNA in BEL-7402 AMD cells, the pro-apoptotic effect of VER was partly reversed, when compared with controls. The apoptotic rate was 15.8% and 18.8% in BEL-7402 (ADM+VER) group and BEL-7402 si-UCHL1 (ADM+VER) group, respectively. Collectively, these results suggest that upregulation UCHL1 could enhance the pro-apoptotic effect of VER in ADM resistant HCC cells.

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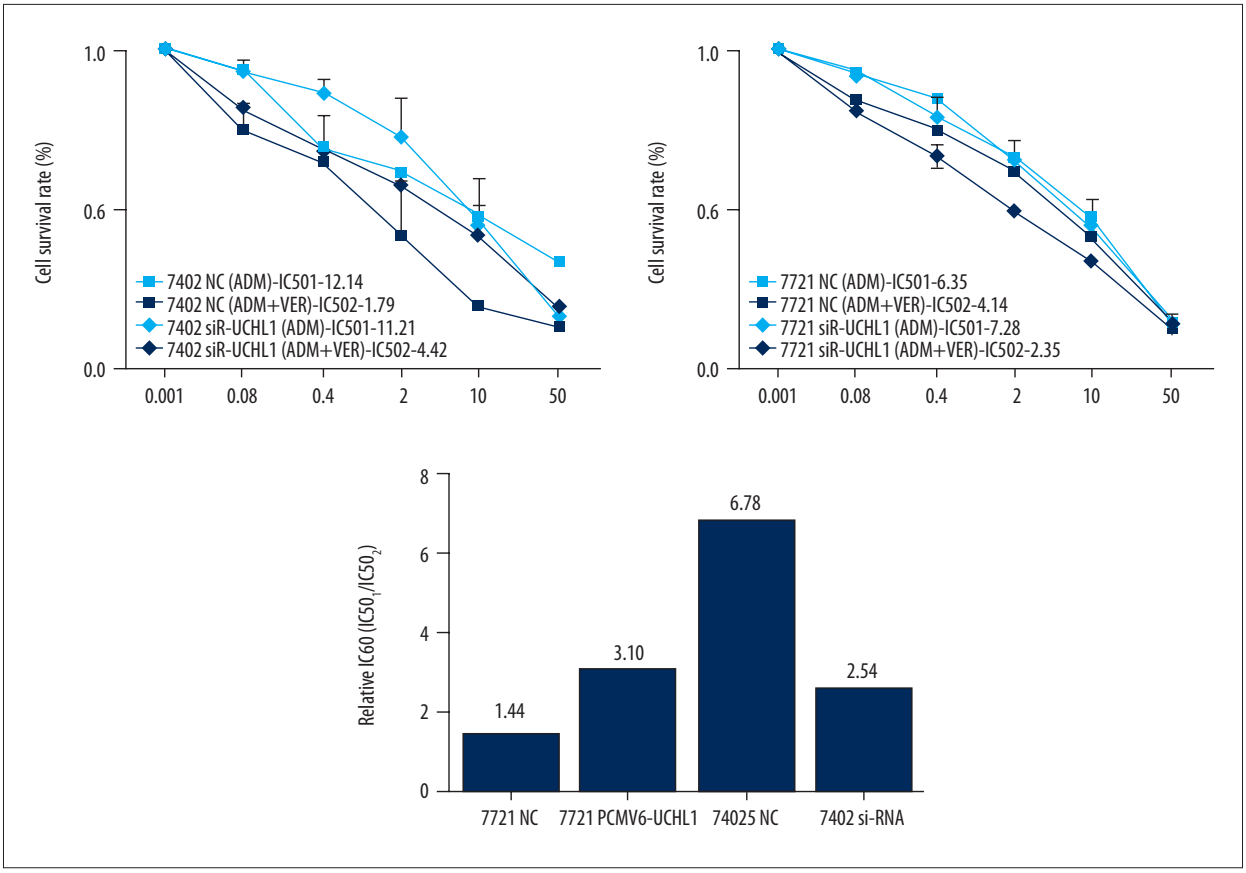

Figure 6. Manipulation of UCHL1 expression with siRNA or overexpression vector in HCC cells, then CCK-8 detects the IC50 of ADM or ADM+VER against HCC cells. UCHL1 – ubiquitin carboxyl-terminal hydrolase L1; HCC – hepatocellular carcinoma; ADM – Adriamycin; VER – verapamil; IC50 – half maximal inhibitory concentration.

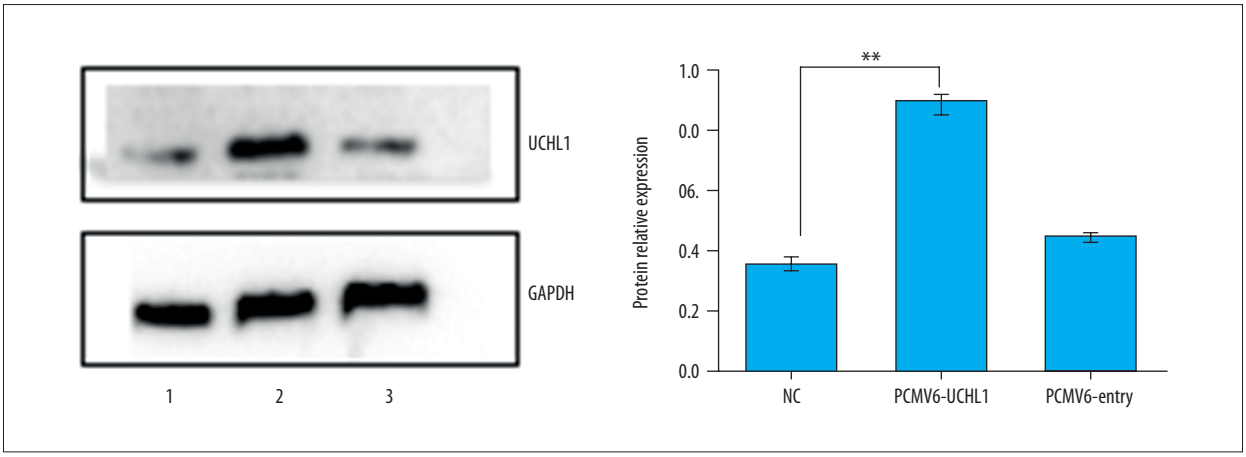

Figure 7. Overexpression UCHL1 in SMMC-7721 cells and western blotting validates the transfection efficiency. UCHL1 – ubiquitin carboxyl-terminal hydrolase L1.

Discussion

VER is a calcium channel blocker. P-glycoprotein (P-gap), also known as multidrug resistance protein 1 (MDR1), has been considered the primary target that mediates the reversal effect of VER on chemo-resistance [12]. P-gp, a trans-membrane protein belonging to the ATP-binding cassette (ABC) family, could transport drugs in the cells to the outside, leading to the chemo-resistance of cancerous cells to chemotherapeutic agents. Previous studies have showed that VER could interfere with the

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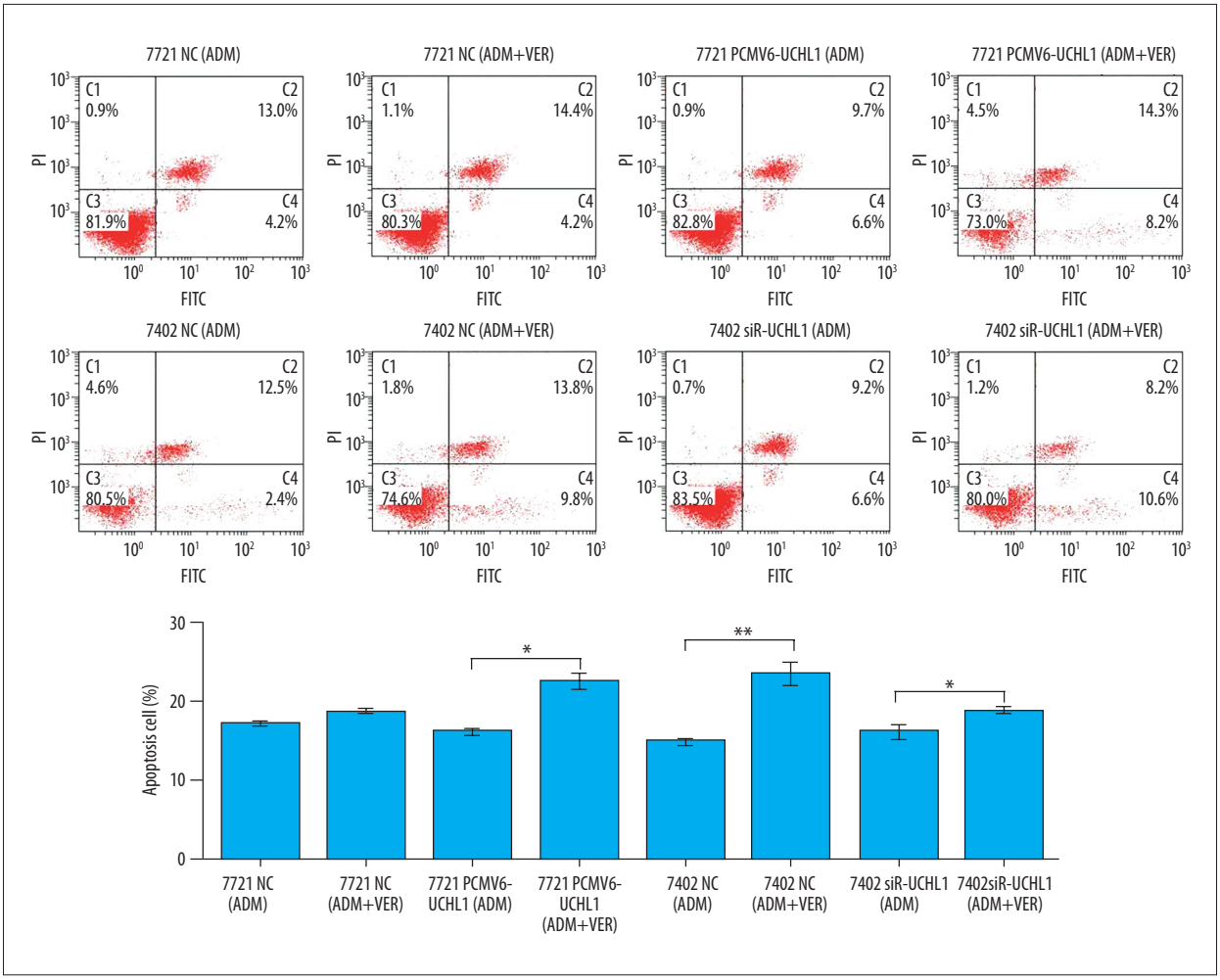

Figure 8. Flow cytometry detects cell apoptosis after transfection UCHL1 siRNA and overexpression vector in ADM or ADM+VER HCC cells. ADM, UCHL1, ubiquitin carboxyl-terminal hydrolase L1; HCC – hepatocellular carcinoma, ADM – Adriamycin; VER – verapamil.

coupling of the transporting capability of P-gp with the energy released by ATP hydrolysis (also known as invalid ATP hydrolysis) and make P-gp lose transportation capability; or VER compete with drugs for the binding site on P-gp, which limits the efflux of drugs [13] or the combination of VER with chemotherapeutic agents could repress the expression of P-gp [14]. However, MDR1 did not show aberrantly high expression in paclitaxel-resistant ovarian cancer cells and ADM-resistant gastric cancer cells [15,16]. Ly et al. reported that the reversal effect of VER on multiple-resistance of non-small cell lung cancer cells were not associated with modulation of P-gp [17]. Our previous work demonstrated that the expression of P-gp was not simply associated with the reversal effect of VER on chemo-resistance of HCC [9], suggesting novel targets might be responsible for the reversal effect of VER on chemo-resistance of HCC.

UCHL1, a member of ubiquitin carboxyl terminal esterase family, is a deubiquitin enzyme [18]. Accumulating evidence shows that UCHL1 is aberrantly expressed in human malignancies and plays a role in the development and progression of a variety of cancers. However, UCHL1 has been shown to have distinct functions in human malignancies. In non-small cell lung cancer and renal cell carcinoma, UCHL1 functions as an oncogene and promotes cell proliferation and migration [19,20]. In contrast, UCHL1 was found to play a role as a tumor suppressor and suppress cell proliferation and induce apoptosis in breast cancer and prostate cancer [21,22]. In breast cancer, UCHL1 was shown to be involved in the regulation of EGFR expression and mediate chemo-resistance [23]. However, whether UCHL1 mediates the reversal effect of VER on chemo-resistance of HCC remains unknown.

In our previous study, we tested the reversal effect of VER on chemo-resistance to L-OHP, ADM, and 5-FU in 4 HCC cell lines (SMMC-7721, BEL-7402, HepG2, and QGY-7703). Our results showed that the reversal effect of VER against chemo-resistance

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was more potent in BEL-7402 cells than in SMMC-7721 cells. Therefore, these 2 cell lines were chosen as model cell lines. High-throughput transcriptome sequencing based on Illumina technology was used to screen whether the candidate genes mediate the reversal effect of VER on chemo-resistance. Among these genes, we postulated that UCHL1 may be the candidate gene that mediates the reversal effect of VER on chemo-resistance [9]. In this study, the mRNA and protein of UCHL1 was examined by qRT-PCR and western blot, respectively. The results demonstrated that the expression of UCHL1 did not show significant differences between the SMMC-7721ADM group and the SMMC-7721ADM+VER group. The expression of UCHL1 in the BEL-7402ADM group and the BEL-7402 ADM+VER group was significantly elevated (** $P<0.01$), which indicated that upregulation of UCHL1 could enhance the reversal effect of VER on chemo-resistance to ADM in HCC. Immunohistochemistry assay of clinical samples also showed that the tissue sample from patients with a positive response to VER treatment exhibited a significantly higher expression of UCHL1, compared with that from patients with a negative response to VER treatment.

To further verify the role of UCHL1 in the reversal effect of VER on chemo-resistance, BEL-7402 cells were transfected with UCHL1 siRNA. The IC_{50} values of BEL-7402 cells and BEL-7402 cells with UCHL1 knockdown of ADM and ADM+VER in ADM and ADM+VER groups were examined using CCK-8 assay. Results showed that the reversal effect of VER was significantly dampened in BEL-7402 cells with UCHL1 knockdown.

In SMMC-7721 cells, UCHL1 was overexpressed and the IC_{50} values of SMMC-7721 cells and SMMC-7721 cells with UCHL1 overexpression in ADM and ADM+VER groups were examined using CCK-8 assay. Results showed that the reversal effect of VER was significantly enhanced in SMMC-7721 cells with UCHL1 overexpression. Taken together, these results showed that UCHL1 was involved in the reversal effect of VER on chemo-resistance of HCC and upregulation of UCHL1 enhanced the reversal effect of VER on chemo-resistance.

Moreover, flow cytometry assay revealed that modulating the expression of UCHL1 could significantly change the promoting effect of VER on ADM-induced apoptosis: upregulation of UCHL1 could enhance the promoting effect of VER on ADM-induced apoptosis while downregulation of could compromise the promoting effect of VER on ADM-induced apoptosis. These results indicated that VER in combination with ADM could promote the cell apoptosis, and UCHL1 is involved in the promoting effect of VER on ADM-induced apoptosis.

Conclusions

Our results showed that UCHL1 upregulation by VER enhanced the reversal effect of VER on chemo-resistance of HCC and promoted cell apoptosis. Based on these results, we will explore in future studies the relevant mechanisms and the molecular signaling pathways.

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