Establishment of a cisplatin-induced multidrug resistance cell line SK-Hep1/DDP

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[Abstract] Background and Objective: Multidrug resistance (MDR) is a major obstacle in the chemotherapy of cancer patients. The aim of this study was to establish a multidrug-resistant cell line SK-Hep1/DDP and explore its molecular mechanism of the MDR. Methods: SK-Hep1/DDP cell line was induced by pulse treatment using a high concentration of cisplatin (DDP) in vitro. The chemoresistance indexes of cells were evaluated by CCK-8 assays. The protein of MDR1 (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2) and Bax were detected by Western blotting, and the effect of MDR1 inhibitor cyclosporine A (CsA) on expression of MDR1 proteins in SK-Hep1 and SK-Hep1/DDP cell lines. Flow cytometry was performed to determine the distribution of the cell cycle and cell apoptosis ratio. Results: The SK-Hep1/DDP cells were 13.76 times more resistant to DDP in comparison with SK-Hep1 cells, and SK-Hep1/DDP cells also exhibited cross-resistance to many other chemotherapeutic agents (adriamycin and 5-fluorouracil). MDR1, MRP1, and MRP2 protein expressions were significantly higher in the SK-Hep1/DDP than in the SK-Hep1 (P < 0.01), but Bax was lower in the SK-Hep1/DDP than in the SK-Hep1 (P < 0.01). There was no obvious influence between SK-Hep1 and SK-Hep1/DDP cells in the expression of MDR1 by MDR1 inhibitor CsA (P > 0.05). The percentages of cells in G0/M and S phase were significantly increased in SK-Hep1/DDP in comparison with those in SK-Hep1 [(20.67 ± 5.69)% vs. (12.14 ± 3.36)%; (42.20 ± 2.65)% vs. (27.91 ± 2.16)%; P < 0.01]. After the cells were exposed to 10 μg/mL DDP for 24 h, the cell apoptosis rate of SK-Hep1/DDP was decreased in comparison with SK-Hep1, but it was increased in those with pretreatment of MDR1 inhibitor CsA as compared with those without pretreatment. Conclusions: A reliable multi-drug resistant human hepatoma cell line SK-Hep1/DDP is successfully established. The MDR mechanisms of this cell lines are closely related to the overexpression of MDR1 MRP1 and MRP2, lower expression of Bax and the attenuated cell apoptosis induced by chemotherapeutic agents.

Key words: Hepatoma, cisplatin, multidrug resistance, SK-Hep1 cell line, MDR1, MRP1, MRP2

Hepatocellular carcinoma (HCC) is one of the most common gastrointestinal tumors, with a high degree of malignancy and poor prognosis. The low rate of resection rate and high recurrence in HCC implies that chemotherapy plays an important role in the comprehensive treatment. Multidrug resistance (MDR) is a major obstacle to the effective treatment of HCC. Although the mechanism of MDR is still unclear, there are two classical types of transporter proteins at the cellular surface which are responsible for MDR in tumors. One is the adenosine triphosphate-binding cassette (ABC) transporter superfamily, which is an energy dependent efflux pump with the function of extruding chemotherapeutic drugs from the cancer cells. The other is the solute carrier transporter superfamily, which can mediate the absorption of intracellular anti-cancer drugs, and drug resistance may result from decreased activity of these transporters. Once the multidrug resistance had been overcome, significant progresses would achieve in chemotherapeutic efficacy. One of the important tools utilized in the study of mechanisms of chemotherapy resistance are established drug resistant cell line in vitro. Therefore, in this study, we established a human hepatoma cell line SK-Hep1/CDDP to explore the biological characteristics and changes of resistant phenotypes. These cell lines can be used as an ideal model for in-depth study of cancer treatment and the reversal of multidrug resistance.

Materials and methods

Materials
Human hepatoma cell line SK-Hep1 was obtained from Cell
Culture Centre of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). High-glucose Dulbecco's Modified Eagle's medium (DMEM/H) and FBS were from Gibco (Gaithersburg, USA). Cell counting kit-8 (CCK-8) was from Dojindo Molecular Technologies (Dojindo, Japan). Adramycin and cisplatin were from Sigma (St Louis, USA). 5-Fluororacil was from Shanghai Xudong Haipu Pharmaceutical Co., Ltd (Shanghai, China). Vincristine was from Guangdong Lingnan Pharmaceutical Co., Ltd (Guangdong, China).

Cyclosporine (CsA) was from Novartis Pharmaceutical Corporation (Basel, Switzerland). Annexin V-FITC/PI apoptosis detection kit was from Nanjing KeyGen Biotech Co., Ltd (Nanjing, China). PI and RNase were from Amresco Company (Amresco, USA). Mouse anti-human Bax, mouse anti-human -actin, mouse anti-human MDR1 (sc-73354) and mouse anti-human MRP1 (sc-18835) monoclonal antibodies were from Santa Cruz (CA, USA). Rabbit anti-human MRP2 polyclonal antibody and HRP-secondary antibody were from Beijing Zhong Shan Golden Bridge Biotechnology Co., Ltd (Beijing, China).

**Methods**

**Cell culture and establishment of SK-Hep1/DDP cell line**

SK-Hep1 cells were cultured in DMEM/H containing 10% FBS, 100 u/mL penicillin and 100 µg/mL streptomycin at 37°C in humidified incubator with atmosphere of 5% CO₂. Trypsin (0.25%) was used for digestion. SK-Hep1 was selected by a high DDP concentration (the final concentration was 0.5 µg/mL), for 24 h, washed with PBS and recultured in drug-free medium. Medium was changed after 1–2 days to wash out dead cells, when cells growing exponentially they were pulsed with 5 µg/mL of DDP. This cyclic treatment was repeated six times over a period of six months. The cell lines were cultured in 0.01 µg/mL of DDP, thus generating the DDP-resistant subline. Before experiments, SK-Hep1/DDP cells were cultured in a DDP-free medium.

**Cell growth assay**

SK-Hep1 and SK-Hep1/DDP cells (5 x 10⁴) were seeded into 24-well plates in DMEM/H containing 10% FBS at 37°C in humidified atmosphere of 5% CO₂ incubator. Count the duplicate plates every 24 h to determine cell proliferation, Cell viability was determined by trypan blue staining and counted on a hemocytometer. Experiments were conducted five days. The doubling time was calculated according to Patterson formula: \( T_d = \frac{T \times \log_2}{(\frac{N_2}{N_1} - \log N_2)} \), \( N_2 \) stands for starting time and \( N_t \) for cell number of \( T \) hours later. \( T \) is the time between \( N_1 \) and \( N_t \) (h).

**CCK-8 assay**

According to the CCK-8 kit manufacturer's instruction, WST-8 method was used to determine drug sensitivity. In brief, 1 x 10⁴/mL cells were seeded as a suspension (200 µL/well) in 96-well plates, after 24 h incubation period, 10-fold diluted five concentrations of DDP, DOX, VCR and 5-FU (original concentrations were DDP 0.05 µg/mL, DOX 0.001 µg/mL, VCR 0.001 µg/mL, and 5-FU 0.025 µg/mL, respectively) were added to the cells. Each concentration was repeated five times. The same volume of PBS served as control, and blank group was used. The cells were incubated at 37°C in the continuous presence of the drug for 24 h. Cell viability was assayed by adding 20 Twenty µL of CCK-8, and cells were cultured for another 2 h. Absorbance value was measured by enzyme linked immunosorbent assay (measurement wavelength was 450 nm and reference wavelength was 650 nm). Relative inhibitory rate was calculated. Relative inhibitory rate (%) = (1 - sample ext / control ext) x 100%. The IC₅₀ value is defined as the dosage of drugs in which 50% of cellular death (50% reduction of absorbance at 450 nm) after 24-hour treatment. Resistant index (RI) = IC₅₀ of SK-Hep1/DDP / IC₅₀ of SK-Hep1.

**Cell cycle analysis**

The SK-Hep1 and SK-Hep1/DDP cells (synchronized) were harvested and washed with PBS, then cells were centrifuged and resuspended with 0.3 mL of PBS, transferred to 1.5 mL EP tubes. Ice-cold ethanol 0.7 mL was added for fixation. Cells were incubated with 1 g/L of RNase at 37°C for 30 min and stained with 100 mg/L PI for 30 min in dark. Samples were analysed for DNA content by FACScan flow cytometer (Becton Dickinson).

**Detection of MDR1, MRP1, MRP2, and Bax expression by Western blotting**

SK-Hep1 cells and SK-Hep1/DDP cells were lysed and supernatants were collected. BCA method was used to determine the protein concentration. After boiling, 20 µg of total protein was separated by 10% SDS-PAGE. Proteins were transferred to PVDF membrane (Roche) by semi-dry transfer method. After blocking with 5% skim milk in TBST (TBS contained 0.05% of Tween-20) at room temperature for 1 h, then incubated with first antibodies (MDR1, MRP1, MRP2, and Bax) (dilutions were 1:500, 1:500, 1:1000, and 1:1000) at 4°C overnight. Membrane was then washed by TBST (TBS contained 0.05% of Tween-20), and incubated with HRP-secondary antibody at 37°C for 1 h. After washing the membrane with TBST, ECL was added to the membrane, developed and fixed for 5 min. Results were recorded. The absorbency of the belts was analyzed by Gel-Pro gel analysis software (Bio-Rad). The experimental data was indicated by the ratio of the density of target protein to the density of β-actin. Each experiment was repeated three times, and the average value was taken as the final results.

**Detection of cell apoptosis index by Annexin V/PI double staining**

Annexin V/PI double staining and FCM were used to detect cell apoptosis. Experiments were divided into five groups: SK-Hep1 group: parental cells treated without DDP; SK-Hep1/DDP group: DDP-resistant cells treated without DDP; SK-Hep1 + 10 µg/mL DDP group: SK-Hep1 cells treated with 10 µg/mL DDP for 24 h; SK-Hep1/DDP + 10 µg/mL DDP group: SK-Hep1/DDP cells treated with 10 µg/mL DDP for 24 h; and SK-Hep1/DDP + 10 µg/mL DDP + 1 µg/mL CsA group: SK-Hep1/DDP cells treated with 10 µg/mL DDP and 1 µg/mL of CsA for 24 h. Cells (1 x 10⁴) were seeded onto 6-well plates and according to different treatment of the groups, each treatment was repeated three times. After 24 h, cells were trypsinized, and adjusted the concentration to 1 x 10⁵ cells/mL, then centrifuged at 1 500 rpm for 5 min. Supernatant was discarded, 5 µL of AnnexinV and 195 µL of buffer and 20 µL of PI were added and cells were incubated in dark for 20 min. Cells (5 x 10⁴) were collected for flow cytometry. The excitation wavelength was 488 nm. FITC was excited to green fluorescence and PI was excited...
to red fluorescence. The fluorescence emitted by cells (10000 cells/sample) was analyzed using a FACSscan flow cytometer (Becton Dickinson). Results were analyzed by Cellquest software. SK-Hep1 and SK-Hep1/DDP groups served as control.

**Statistical analysis**

The data were collected and analyzed by SPSS, and data are expressed as mean ± standard deviation (SD). Differences between two groups were evaluated by one-factor analysis of variance (ANOVA) and t test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Establishment of multidrug resistant cell line SK-Hep1/DDP**

In the process of pulse treatment using high-dose DDP, most of the cells were enlarged in cell volume, and many vacuoles appeared in cytoplasm and vague cell membrane. Cells gradually died and floated. A few cells without drug treatment survived after a period of time, and resumed to logarithmic growth. We established the human hepatoma cell line SK-Hep1/DDP after six months with stable resistance to DDP. These cells had spindle like morphology and elongated easily, and cell protrusion and black particles in the cytoplasm increased. The cell morphology was close to the parent cells. The resistance index of DDP in SK-Hep1/DDP cells was 13.76 by CCK-8 assay. A series of MDR-sublines SK-Hep1/DDP was established successfully. The growth curve is shown in Figure 1. The cell growth rate of SK-Hep/DDP cells was slower than parental cells, and the population doubling time extended in SK-Hep1/DDP cells. The doubling times were \( (25.41 \pm 0.39) \) h and \( (30.56 \pm 0.59) \) h, respectively in two cell lines, with statistically significant differences (\( P < 0.05 \)).

![Figure 1](image1.png)

**SK-Hep1/DDP multidrug resistance**

The \( IC_{50} \) DDP in SK-Hep1 and SK-Hep1/DDP cells were 5.13 ± 0.09 and 70.61 ± 1.06, respectively. The drug resistance index was 13.76 in SK-Hep1/DDP cells. We further tested the drug resistant effects on SK-Hep1 and SK-Hep1/DDP cells induced by other chemotherapeutic drugs (Table 1). The results showed that drug resistant cell line SK-Hep1/DDP displayed cross-resistance to these drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>( IC_{50} ) (( \mu )g/mL)</th>
<th>Resistance index</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP</td>
<td>5.13 ± 0.09</td>
<td>70.61 ± 1.06</td>
</tr>
<tr>
<td>DOX</td>
<td>0.74 ± 0.04</td>
<td>4.13 ± 0.23</td>
</tr>
<tr>
<td>VCR</td>
<td>0.76 ± 0.02</td>
<td>2.28 ± 1.06</td>
</tr>
<tr>
<td>5-FU</td>
<td>12.49 ± 0.27</td>
<td>52.79 ± 3.85</td>
</tr>
</tbody>
</table>

All data are presented as mean ± standard deviation. \(^* P < 0.01, \) vs. SK-Hep1 cells.

**Cell cycle analysis by flow cytometry**

In SK-Hep1 cells, the percentage of cells was \( (59.83 \pm 3.28)\% \) in G1 phase \( (27.91 \pm 2.16)\% \) in S phase, and \( (12.14 \pm 3.36)\% \) in G2/M phase. In SK-Hep1/DDP cells, the percentage of cells was \( (37.50 \pm 5.05)\% \) in G1 phase, \( (42.20 \pm 2.65)\% \) in S phase and \( (20.67 \pm 5.69)\% \) in G2/M phase. Obviously, in comparison with parental SK-Hep1 cells, the percentage in G2/M and S phases increased in drug resistant SK-Hep1/DDP cells, while it decreased in G1 phase (\( P < 0.01 \)).

**Western blot**

The expression of MDR1, MRP1, MRP2, and Bax were detected in both SK-Hep1 and SK-Hep1/DDP cells (Table 2 and Figure 2) to further characterize the resistance and its mechanisms. Western blot analysis confirmed that the MDR1, MRP1, and MRP2 expressions (gray value was proportional to the level of protein expression) were significantly increased in SK-Hep1/DDP cells (\( P < 0.01 \)), while the Bax expression was lower than in parental cells (\( P < 0.01 \)).

![Figure 2](image2.png)

**Effects of MDR Inhibitor CsA on MDR expression in human hepanoma cells**

We examined the expression of MDR1 in both SK-Hep1 and...
SK-Hep1/DDP cells, and found that MDR1/β-actin ratio was 0.58 ± 0.02 and 1.32 ± 0.04 in two cell lines, respectively. SK-Hep1 cells and SK-Hep1/DDP cells were exposed to MDR1 inhibitor CsA (1 μmol/L) for 24 h, and MDR1/β-actin ratio was 0.54 ± 0.06 and 1.24 ± 0.06 in two cell lines. Comparing with the endogenous MDR1 expression in the two cell lines, the differences were not statistically significant (P > 0.05), indicating that CsA did not affect MDR1 expression directly.

**Table 2** The expression levels of four proteins in SK-Hep1 and SK-Hep1/DDP cells

<table>
<thead>
<tr>
<th>groups</th>
<th>MDR1/β-actin</th>
<th>MRP1/β-actin</th>
<th>MRP2/β-actin</th>
<th>Bax/β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-Hep1</td>
<td>0.58 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>0.34 ± 0.05</td>
<td>3.53 ± 0.12</td>
</tr>
<tr>
<td>SK-Hep1/DDP</td>
<td>1.32 ± 0.04</td>
<td>0.59 ± 0.03</td>
<td>2.18 ± 0.10</td>
<td>0.59 ± 0.03</td>
</tr>
</tbody>
</table>

All data are presented as mean ± standard deviation. *P < 0.01, vs. SK-Hep1 cells.

Figure 3 Western blotting for effects of cyclosporine (CsA) on MDR1 in SK-Hep1 and SK-Hep1/DDP cells
Lane 1, SK-Hep1; lane 2, SK-Hep1/DDP; lane 3, SK-Hep1 + 1 μmol/L CsA; lane 4, SK-Hep1/DDP + 1 μmol/L CsA.

**Cell apoptosis analysis by Annexin V/PI double staining**

The natural cell apoptosis rates were (2.00 ± 0.11)% and (0.67 ± 0.08)% in SK-Hep1 cells and SK-Hep1/DDP cells as shown by flow cytometry. After exposed to 10 μg/mL DDP treatment for 24 h, the cell apoptosis rates increased to (56.3 ± 2.9)% in SK-Hep1 cells, and to (61.7 ± 0.11)% in SK-Hep1/DDP cells. Therefore, SK-Hep1/DDP cells were more resistant to DDP than SK-Hep1 cells (P < 0.01). After treatment with MDR inhibitor CsA, the cell apoptosis rate caused by DDP was increased to (27.8 ± 1.6)% in SK-Hep1/DDP cells (P < 0.01).

**Discussion**

Multidrug resistance is still a major obstacle to cancer therapy, which is also the main reason for the failure of HCC chemotherapy. The establishment of drug resistant cancer cell lines in vitro could provide an important means and model for studying the mechanism of MDR and the reversal of resistance. Additionally, the establishment of drug resistant cancer cell lines in vitro can enhance the screening of chemotherapeutic drugs, improve the sensitivity of cancer cells to chemotherapeutic drugs and provide a model of reversing drug resistance, thus overcoming the obstacles in clinical chemotherapy. Once the tumor multidrug resistance had been overcome, significant progress would be made in chemotherapeutic efficacy.

As a tool for studying the mechanism of tumor multidrug resistance, the establishment of drug resistant cell lines in vitro has a history of more than 20 years. Generally, high-dose intermittent induction or increased concentration are used in parental cells to establish the cell lines. Intermittent high-dose induction is similar to clinically periodic chemotherapy, therefore, can simulate the drug resistant phenomena in patients after chemotherapy, which is more representative. DDP is a traditional anti-cancer drug used clinically. Except for direct cytotoxicity, DDP also induces cell apoptosis. In this study, we used human hepatoma cell line SK-Hep1 as experimental object to establish DDP resistant cell line SK-Hep1/DDP and explored the mechanism of drug resistance.

In the present study, we successfully established DDP resistant cell line SK-Hep1/DDP by intermittent high-dose DDP-induced method for six months in vitro screening, the resistance index was 13.76. As shown from the results of cell growth curve and cell doubling time, the proliferation rate of SK-Hep1/DDP was decreased, and the population doubling time was extended as compared with parental cells SK-Hep1. The cell cycle analysis found that the percentage of G2/M and S phase cells in SK-Hep1/DDP cells was higher and that of G1 cells was lower than in parental SK-Hep1 cells (P < 0.01). SK-Hep1/DDP was not only susceptible to DDP, but also caused different degrees of cross-resistance to never touched DOX, VCR, 5-FU, and other anti-cancer drugs with different mechanisms. The establishment of multi-drug resistant cell line SK-Hep1/DDP has provided a powerful tool for further study of the multi-drug resistant phenotypes.

The mechanism of MDR was complicated, including increased drug efflux, decreased drug absorption, altered anti-cancer drugs targets, reduced drug activation, enhanced DNA damage repair capacity, inhibited cell apoptosis and so on. Among them, decreased drug concentration in vivo was the most common phenomenon of weakening the effects of anti-cancer drugs. Transporter proteins associated with the mechanism of multidrug resistance, including ABC transporter superfamily, such as the increased expression of MDR1/ABC1, MRP1/ABCC1 and BCRP/ABCG2. MRP2/ABCC2 was over-expressed in ovarian cancer, liver cancer and other DDP resistant cells. Tumor cells accessed the multidrug resistance while the ability of anti-apoptosis induced by chemotherapeutic drugs enhanced, indicating multidrug resistance of tumor cells was closely related to apoptosis inhibition. For this reason, we examined the expression of MDR1, MRP1, MRP2 and pro-apoptotic protein Bax in SK-Hep1 and SK-Hep1/DDP cells and detected the differences between the two cell lines. Our results showed that in DDP resistant cell line SK-Hep1/DDP, the expression of MDR1, MRP1 and MRP2 were significantly increased. Especially the MRP2 expression in DDP-resistant SK-Hep1/DDP cells, which was 6.4 times higher than in sensitive cells, while the apoptotic protein expression significantly reduced. Our results confirmed that the
Drug resistance of SK-Hep1/DDP cells was positively related to MDR, MRP1 and MRP2 expression, and the expression of pro-apoptotic protein Bax reduced.

Though there are different mechanisms and targets in clinical cancer therapy, all the chemotherapeutic drugs lead to cell apoptosis finally. Under the induction of chemotherapeutic drugs, tumor cells usually produced apoptotic tolerance. Therefore, we further examined the apoptosis rate of human hepatoma cells induced by cisplatin by flow cytometry. The results showed that the apoptosis/death rate in SK-Hep1/DDP cells caused by DDP decreased significantly as compared with SK-Hep1 cells. However, whether the reduction of pro-apoptotic effects was related to the expression or activation of MDR1 is not clear. We further treated SK-Hep1/DDP cells with cyclosporine A and DDP, and found that cyclosporine A enhanced the apoptotic effects by DDP on SK-Hep1/DDP cells. Cyclosporin A is an immunosuppressive drug, which is a broad-spectrum MDR-related regulator. Except for affecting the activity of MDR1/ABCB1, cyclosporine A also affected MRP and BRCP activities, but did not affect MDR1 expression. Our data also found that after exposing to low dose CsA, MDR1/ABCB1 expression in SK-Hep1 and SK-Hep1/DDP cells was not statistically different in comparison with non-treatment groups (P > 0.05), suggesting that low-dose CsA did not inhibit MDR1/ABCB1 expression, which may play a regulatory role in affecting MDR1/ABCB1 activity. Thus, the results of CsA treatment confirmed that the drug resistance of SK-Hep1/DDP cells was closely related to MDR1, MRP1 and MRP2 expression, and prompted cell apoptosis may be involved in the formation of multidrug resistance.

In summary, this study has for the first time established a new human hepatoma SK-Hep1/DDP. The drug resistant mechanism may be related to inducing over-expression of MDR1, MRP1 and MRP2, and reducing the pro-apoptotic effects on tumor cells by anti-cancer drugs.

References