

Silence of p15 expression by RNAi enhances cisplatin resistance in hepatocellular carcinoma cells

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ABSTRACT

The insensitivity of hepatocellular carcinoma to chemotherapy is associated with alternation in tumor cell cycling. This current study was designed to investigate the impact of p15 silencing on the sensitivity of Human hepatocellular carcinoma HepG2 cells to cisplatin.

HepG2/CDDP/1.6 and HepG2/CDDP/2.0 cells were induced by culture with increased doses of cisplatin and their sensitivities to cis-Diamine dichloroplatinum (CDDP) were determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The impacts of p15 silencing on the cell cycling and P-gp expression were characterized by flow cytometry, RT-PCR and Western blot assays, respectively. Knockdown of p15 expression dramatically reduced the relative levels of p15 expression and the frequency of phase G₁, promoting cell cycling. On the other hand, knockdown of p15 expression significantly up-regulated the expression of P-glycoprotein (P-gp) in HepG2/CDDP/2.0 cells, associated with the increased resistance of HepG2 cells to CDDP *in vitro*.

In conclusion, the p15 may be a critical regulator of the development of CDDP resistance in HepG2 cells.

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KEY WORDS: hepatocellular carcinoma, cell cycle, p15, RNA interference, drug resistance

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most malignant tumors worldwide [1], and is generally insensitive to anticancer drugs, which makes chemotherapeutic drug treatment difficult. It is estimated that 70% of patients who have received chemotherapeutic drugs rapidly developed drug resistance [2, 3], which remains a difficult issue. Therefore, discovery of mechanisms underlying the drug resistance is of great significance in the development of new strategies for the intervention of drug-resistant tumors. The development of drug resistance is attributed to the expression of drug-resistance related genes in tumor cells. A previous study has revealed that various cellular pathways may be involved simultaneously in the drug resistance [4]. Increased repair of drug-induced DNA damage, blocked apoptosis, disruptions in the signal pathways, and alterations of factors involved in cell cycling contribute to the development of drug resistance [5, 6]. The cell cycle represents a se-

ries of tightly integrated events that allow the cells to grow and proliferate. The cyclin dependent kinases (CDKs) are important components of the cell cycle machinery, and are positively regulated by cyclins and negatively regulated by cyclin dependent kinase inhibitors (CDKIs) [7]. p15, one member of the cyclin-dependent kinase inhibitors (CDKIs) family, can prevent transition from the G₁ to S phase by inhibiting CDK4/6-cyclinD mediated phosphorylation of retinoblastoma (Rb) [8]. Induction of p15 over-expression can induce G₁-phase arrest through the TGF- β /Smad pathway [9]. Indeed, down-regulated expression of p15 and related signaling that control G₁/S transition are frequently deregulated in human cancers, such as pituitary adenoma, myeloid disease, and gastric cancer [10-12]. Furthermore, loss of p15 expression is associated with the development of lymphoproliferative disorders and tumor formation [13]. Consequently, targeting the cell cycle modulators, CDKI, may be an excellent opportunity for the treatment of hepatocellular carcinoma [10]. Our previous study has shown that up-regulating p15 expression inhibits the progression of cell cycling and reverses the drug-resistant phenotype in drug-resistant HepG2/CDDP/2.0 cells [14]. This study is aimed at examining the impact of p15 knockdown on the progression of cell cycling and resistance to CDDP in CDDP-resistant HepG2 cells.

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MATERIALS AND METHODS

Establishment of CDDP-resistant HepG2 cells

Human hepatocellular carcinoma HepG2 cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (CAS, Shanghai, China), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, USA). The cells were exposed to increased doses of cisplatin (0, 0.1, 0.2, 0.4, 0.8, or 1.6 mg/L, Hospira, Unterach, Australia) for three months to generate a cisplatin-resistant HepG2/CDDP/1.6 cell line. Finally, the cells were maintained in 1.6 mg/L of CDDP in complete medium. In addition, HepG2/CDDP/1.6 cells were cultured in the presence of 2.0 mg/L CDDP (designated as HepG2/CDDP/2.0) and were also used for experiments.

MTT assay

The sensitivity of HepG2/CDDP/1.6 and HepG2 cells to CDDP was tested by MTT assays. Briefly, HepG2/CDDP/1.6 and HepG2 cells at 2×10^5 per well were cultured in 10% FBS RPMI 1640 medium in 96-well plates and treated in triplicate with 1.6 or 0 mg/L of CDDP for 24, 48, and 72 h, respectively. During the last 4h incubation, the cells were exposed to 50 $\mu\text{g/mL}$ of MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide, Sigma, St. Louis, USA). The resulting formaza in individual wells was dissolved with 150 μl of DMSO (Sigma, St Louis, USA) and measured at an absorbance of 570 nm using a VERSAmax spectrophotometer (Molecular Devices, Sunnyvale, CA). The cells cultured in medium alone were used as negative controls, and the IC₅₀ for CDDP was calculated using the spass 10.0 software.

Transfection of siRNA

The 21-nucleotide p15-specific siRNA Y1, Y2, and Y3 duplexes or control siRNA-F were designed and chemically synthesized by Sangon Biotech (Shanghai, China), and their sequences are shown in Table 1. To knockdown p15 expression, HepG2/CDDP/2.0 cells at 5×10^5 cells/well were incubated overnight in 10% FBS RPMI 1640 medium in 6-well plates, and transfected in triplicate with different doses (30-90 nM) of the 21-nucleotide p15 specific siRNA Y1, Y2, and Y3 duplexes or control siRNA-F (90 nM), re-

spectively, using Lipofectamine™ 2000, according to the manufacturers' instruction (Invitrogen). The levels of p15 mRNA transcripts were determined 24, 48, and 72 h later for evaluating the efficacy of p15 silencing by RT-PCR.

RT-PCR

HepG2/CDDP/2.0 cells were transfected with different doses of Y1, Y2, Y3, or siRNA-F for 24, 48, and 72 h (as described above), and their total RNA was extracted, respectively, using the RNAiso™ Plus reagent, according to the manufacturers' instruction (TaKaRa Biotechnology, Otsu, Japan). The RNA samples (1 μg /per group) were reversely transcribed into cDNA using the one-step RT-PCR kit (Takara Biotechnology), according to the manufacturer's instructions. The relative levels of p15 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA transcripts were determined by PCR using the cDNA as the template and specific primers. The sequences of primers were sense 5-CGTTAAGTTTACGGCCAACG-3 and anti-sense 5-GGTGAGAGTGGCAGGGTCT-3 for p15 (302bp), and sense 5-GAGCCAAAAGGTCATCATCTC-3 and anti-sense 5-AAAGGTGGAGTGGGTGTC-3 for GAPDH (542bp), respectively. The PCR reactions were performed in duplicate at 94°C for 5 min and then subjected to 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, followed by an extension at 72°C for 10 min. The PCR products were resolved on agarose-gel electrophoresis, and the relative levels of p15 mRNA transcript to GAPDH were determined using the Quantity One Software (Bio-Rad Laboratories, Beijing, China).

Cell cycle analysis

HepG2/CDDP/2.0 cells were transfected in triplicate with the Y3 or control siRNA-F (90 nM) for 24, 48, and 72 h, respectively, and the cells were harvested, respectively. Subsequently, the cells (1×10^6 cells/tube) were washed with pre-cooled phosphate buffered solution (PBS) twice and fixed with ice-cold 70% ethanol, followed by staining with 20 $\mu\text{g/mL}$ of propidium iodide (PI, Sigma). The distribution of individual cell cycle phases was analyzed by flow cytometry using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) for analysis of the DNA contents.

Western blot analysis

HepG2/CDDP/2.0 cells were transfected with Y3 (90 nM) or siRNA-F for 72 h and the cells were harvested, respectively. The cells were lysed in cell lysis buffer for Western blot (Beyotime Institute Biotechnology, Shanghai, China) for 30 min on ice, and their protein concentrations were determined using the Pierce BCA reagents (Pierce, Rockford, USA), according to the manufacturers' instruction. The cell lysates were mixed

TABLE 1. The sequences of the p15-specific siRNAs

Gene	Accession	Sequence
P15-Y1	NM_004936	Sense: 5-CGGAGUCAACCGUUUCGGGUU-3 Antisense: 5-CCCGAAACGGUUGACUCCGUU-3
P15-Y2	NM_004936	Sense: 5-GGGAUUUUAGGAGUGUGUTT-3 Antisense: 5-ACACACUCCUAAAUAUCCCTG-3
P15-Y3	NM_004936	Sense: 5-AAACGGUUGACUCCGUUGGTT-3 Antisense: 5-AAACGGUUGACUCCGUUGGTT-3
SiRNA-F		Sense: 5-UUCUCCGAACGUGUCACGUTT-3 Antisense 5-ACGUGACACGUUCGGAGAATT-3

with SDS-PAGE Sample Loading Buffer (5×) and boiled for 10 min, followed by centrifuging. The cell lysates (50µg/lane) were separated by electrophoresis on 12% SDS-PAGE gel and transferred into PVDF membranes. Subsequently, the membranes were blocked with 5% non-fat milk in TBST with 0.1% Tween 20 for 2 h at room temperature, and then incubated overnight with 2 µg/ml of mouse-anti-human p15 monoclonal antibody and 1:200 diluted mouse-anti-human p-gp polyclonal antibodies (Santa Cruz Biotech, Santa Cruz, USA) at 4°C. After washing, the bound antibodies were detected with 1:20000 diluted peroxidase-conjugated affinity-chromatography pure goat anti-mouse IgG (Santa Cruz Biotechnology) for 1 h at room temperature, and visualized using the enhanced chemiluminescence reagent (Santa Cruz Biotechnology) and Gel Doc™ XR+ Imaging System (Bio-Rad Laboratories). The relative levels of p15 proteins to control GAPDH were analyzed using Image Lab™ 2.0 Software (MCM DESIGN, Copenhagen, Denmark).

Statistical analysis

Data are expressed as mean ± SD of each group. The difference among groups was analyzed by one way of analysis variance (ANOVA), and the difference between groups was determined by Student's *t*-test using Excel 7.0 software. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Establishment of CDDP-resistant HepG2/CDDP/1.6

To determine the impact of modulating p15 expression on the chemo-resistance of HepG2 cells to CDDP, HepG2 cells were cultured with increased doses of CDDP for three months and maintained with 1.6 mg/L of CDDP to generate the CDDP-resistant HepG2/CDDP/1.6. Analysis of its

sensitivity, together with its parent HepG2 cells, to cisplatin revealed that the IC₅₀ values of HepG2/CDDP/1.6 cells to cisplatin was 3.47 ± 0.06 mg/L, and their parent HepG2 cells was 0.51 mg/L ± 0.01 mg/L, resulting in a resistance index of 6.8. HepG2/CDDP/1.6 cells grow slowly *in vitro* to accommodate the context of cisplatin (Figure 1). Hence, the establishment of CDDP-resistant HepG2/CDDP/1.6 cells provides an excellent model for the following studies.

Knockdown of p15 expression in the CDDP-resistant HepG2 cells

To optimize the conditions, HepG2/CDDP/1.6 cells were transfected with 30-90 nM of control siRNA-F for 24 h. As shown in Figure 2, transfection with 90 nM of siRNA-F for 24 h resulted in nearly 95% of cells carrying the siRNA-F. Next, we determined the efficacy of transfection with the p15-specific siRNA on the p15 expression in HepG2/CDDP/2.0 cells. HepG2/CDDP/2.0 cells were cultured overnight with complete medium in the presence of 2.0 mg/L of CDDP, and transfected with 30-90 nM of control siRNA-F or the p15-specific siRNA Y1, Y2, or Y3 for 24-72 h, respectively. As shown in Figure 3A and 3B, while transfection with the siRNA-Y1 did not change the levels of p15 mRNA transcripts, transfection with the siRNA-Y2 obviously reduced the levels of p15 mRNA transcript by 30% in HepG2/CDDP/2.0 cells. More importantly, transfection with the siRNA-Y3 significantly reduced the levels of p15 mRNA transcripts even at 24 h post transfection, and transfection with the siRNA-Y3 for 72 h dramatically minimized the p15 expression by 70%. Similarly, transfection with the siRNA-Y3 inhibited the expression of p15 proteins in HepG2/CDDP/2.0 cells *in vitro* (Figure 3C). Hence, the siRNA-Y3 inhibited the expression of p15 in HepG2/CDDP/2.0 cells in a dose and time-dependent manner.

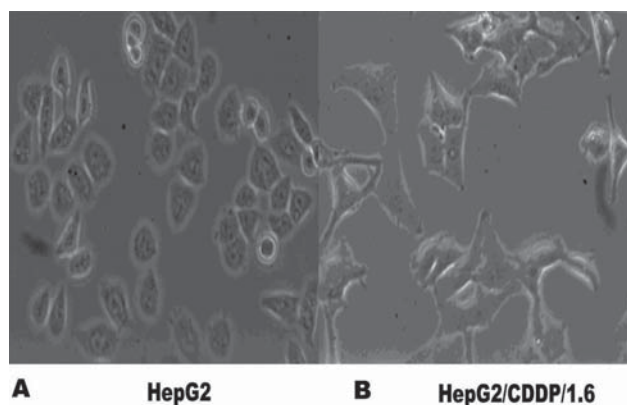


FIGURE 1. The morphology of CDDP-resistant HepG2 cells. HepG2 cells were cultured in the presence of increased concentrations of CDDP for three months and became a CDDP-resistant cell line, HepG2/CDDP/1.6. (A): HepG2; (B): HepG2/CDDP/1.6 cells (200× magnification).

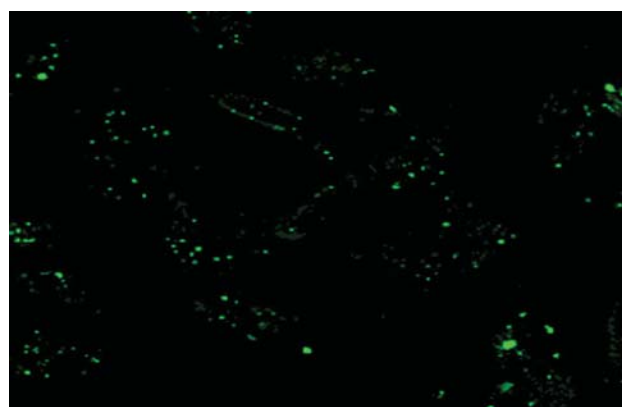


FIGURE 2. Laser scanning confocal microscopy (LSCM) analysis of transfected HepG2/CDDP/2.0 cells. HepG2/CDDP/2.0 cells were transfected with control siRNA-F (90 nM) for 24h and subjected to LSCM analysis. The green dots represent the transfected siRNA (400× magnification).

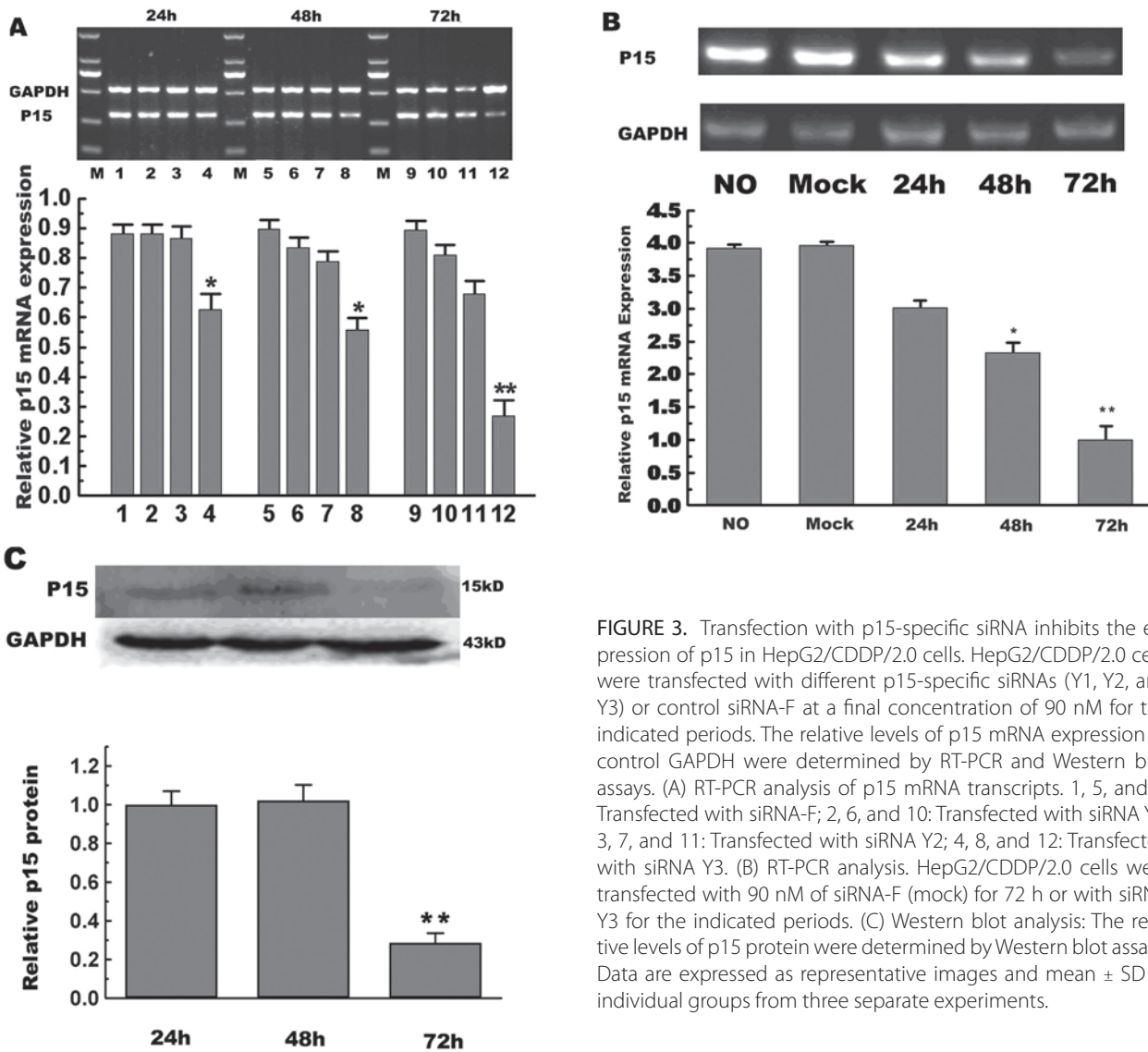


FIGURE 3. Transfection with p15-specific siRNA inhibits the expression of p15 in HepG2/CDDP/2.0 cells. HepG2/CDDP/2.0 cells were transfected with different p15-specific siRNAs (Y1, Y2, and Y3) or control siRNA-F at a final concentration of 90 nM for the indicated periods. The relative levels of p15 mRNA expression to control GAPDH were determined by RT-PCR and Western blot assays. (A) RT-PCR analysis of p15 mRNA transcripts. 1, 5, and 9: Transfected with siRNA-F; 2, 6, and 10: Transfected with siRNA Y1; 3, 7, and 11: Transfected with siRNA Y2; 4, 8, and 12: Transfected with siRNA Y3. (B) RT-PCR analysis. HepG2/CDDP/2.0 cells were transfected with 90 nM of siRNA-F (mock) for 72 h or with siRNA Y3 for the indicated periods. (C) Western blot analysis: The relative levels of p15 protein were determined by Western blot assays. Data are expressed as representative images and mean \pm SD of individual groups from three separate experiments.

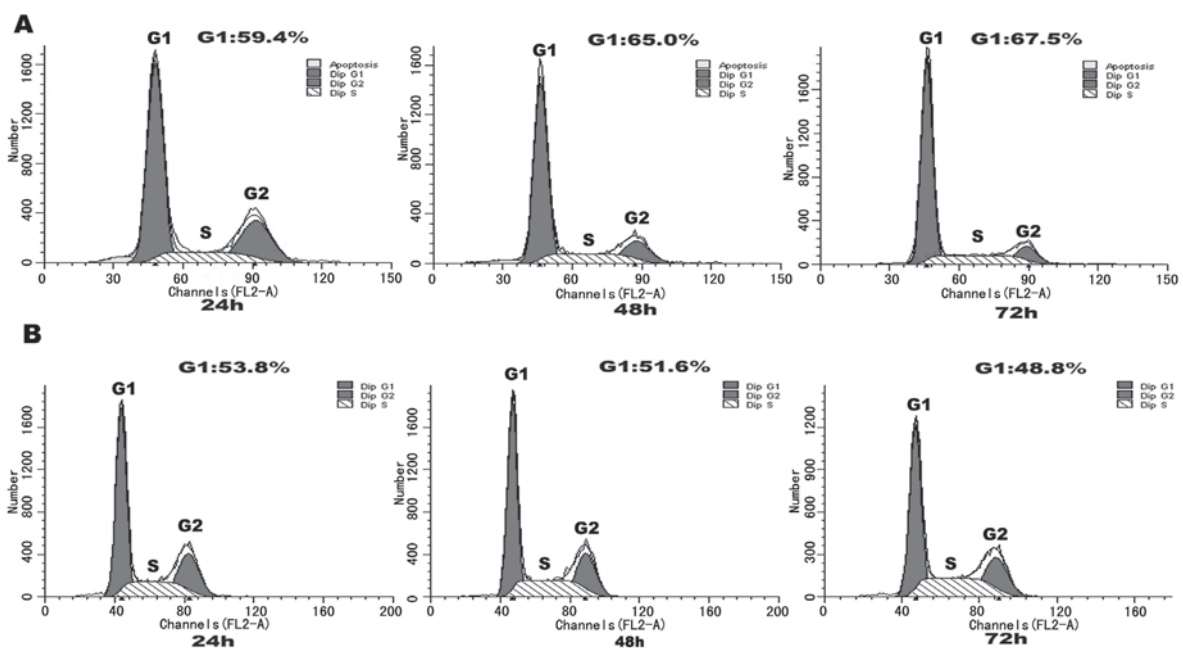


FIGURE 4. Analysis of cell cycle. HepG2/CDDP/2.0 cells were transfected with 90 nM of control siRNA-F (A) or siRNA Y3 (B) for the indicated periods and the distribution of different phases of cell cycling was characterized by flow cytometry.

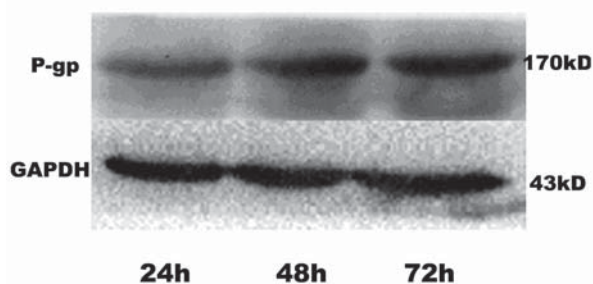


FIGURE 5. Characterization of p-gp expression. HepG2/CDDP/2.0 cells were transfected with 90 nM of siRNA Y3 for varying periods, and the relative levels of p-gp expression to control GAPDH were determined by Western blot assays. Data shown are representative images from three separate experiments.

Knockdown of p15 expression promotes cell cycle progression in HepG2/CDDP/2.0 cells

To determine the impact of p15 silencing on the progression of cell cycling, HepG2/CDDP/2.0 cells were transfected with 90 nM of control siRNA-F or siRNA-Y3 for 24-72 h, respectively. The cells were harvested and their cell cycling was characterized by flow cytometry analysis (Figure 4). The frequency of HepG2/CDDP/2.0 cells that had been transfected with control siRNA-F at G₁ phase increased gradually with the extended culture time. In contrast, transfection with the siRNA-Y3 appeared to promote the progression of cell cycling in HepG2/CDDP/2.0 cells. Evidentially, the frequency of siRN-Y3-transfected HepG2/CDDP/2.0 cells at G₁ phase was reduced to 53.8% at 24 h post transfection and further decreased to 48.8% at 72 h post transfection ($p < 0.05$). Therefore, knockdown of p15 expression promoted the progression of cell cycling in HepG2/CDDP-2.0 cells *in vitro*.

Effect of p15 silencing on the expression of p-gp in HepG2/CDDP/2.0 cells

The increased levels of p-gp expression are associated with drug resistance in tumor cells [15, 16]. Finally, we examined the impact of p15 silencing on the expression of p-gp in HepG2/CDDP/2.0 cells. HepG2/CDDP/2.0 cells were transfected with 90 nM of siRNA-Y3 for 24-72 h, respectively, and the relative levels of p-gp proteins were determined by Western blot assays (Figure 5). Apparently, the levels of p-gp expression increased with extended culture time in the Y3-transfected HepG2/CDDP/2.0 cells, which may be associated with increased drug-resistance to CDDP in HepG2 cells.

DISCUSSION

The change in cell cycling is associated with drug resistance in tumor cells [17]. Notably, CDKs can inhibit the progression of cell cycling and rescue drug resistance in some tumors. In this study, we examined the effect of p15 silencing on the cell cycling and CDDP-resistance in HepG2 cells. We first gen-

erated the CDDP-resistant HepG2/CDDP/1.6 cells *in vitro* by continually culturing HepG2 cells with increased concentrations of CDDP. We found that HepG2/CDDP/1.6 cells grow slowly *in vitro* to accommodate the context of cisplatin. Furthermore, we transfected HepG2/CDDP/2.0 cells with individual siRNA specific targeting p15 and found that transfection with the Y3 siRNA inhibited the p15 expression in a dose and time-dependent manner *in vitro*. More importantly, while transfection with control siRNA did not alter the progression of cell cycling with increased frequency of HepG2/CDDP/2.0 cells at phase G₁, knockdown of p15 significantly reduced the frequency of HepG2/CDDP/2.0 cells at phase G₁, demonstrating that knockdown of p15 promoted the progression of cell cycling in CDDP-resistant HepG2/CDDP/2.0 cells *in vitro*. Finally, we found that knockdown of p15 up-regulated the expression of P-gp in HepG2/CDDP/2.0 cells *in vitro*. These data extend our previous findings that up-regulating p15 expression reverses the drug resistance of hepatocellular carcinoma cells [14]. Given that promotion of cell cycling is associated with drug resistance in tumor cells, our findings suggest that p15 may be a critical regulator of the development of drug resistance in HepG2 cells. The drug-resistant tumor cells usually maintain low concentrations of anti-tumor drug in the cell body, which is associated with an increase in the expression of transporter protein P-gp [18]. The CDDP-resistant tumor cells are insensitive to cisplatin, which can bind to DNA, create adducts and break DNA strand, leading to inhibition of DNA replication. The CDDP-resistant tumor cells usually have sufficient capacity to repair damaged DNA and maintain cell cycling [19]. The cellular response to DNA damage can activate cell cycle checkpoints that serve as natural surveillance mechanisms for DNA integrity. Indeed, the CDDP-resistant HepG2 cells usually exhibit cell cycle arrest at G₀/G₁ phase, a survival mechanism, which provides an excellent opportunity for the tumor cells to repair damaged DNA [7]. We found that the frequency of HepG2/CDDP/2.0 cells in G₀/G₁ phase increased gradually with the increased time in the presence of cisplatin, while the distribution of different phases of cell cycle did not change significantly in stably CDDP-resistant HepG2/CDDP/1.6 cells. The consistent resistance may stem from decreased uptake of cisplatin and/or increased DNA repair [19]. Our data are consistent with a previous observation of cell cycle arrest at phase G₀/G₁ of human QGY/CDDP cells [20]. Our previous study has demonstrated that up-regulation of p15 expression induces cell cycle arrest at G₁ phase and inhibits the cell proliferative activity, reducing drug-resistance in HepG2/CDDP/2.0 cells [14]. In the current study, we found that knockdown of p15 expression promoted cell cycling and increased CDDP-resistance in HepG2/CDDP/2.0 cells. Together, p15 is a negative regulator of cell cycle, associated with

the development of CDDP-resistance in HepG2 cells [10, 21]. RNA interference (RNAi) is a powerful technique to silence gene expression post-transcriptionally, and it can inhibit the mRNA translation and promote mRNA degradation [22]. The RNAi technique has been a promising therapeutic strategy [23]. Chemically synthetic small interfering RNA (siRNA) is currently being evaluated as a potentially useful method for developing highly specific gene-silencing therapeutic reagents [22]. We found that transfection with the Y3 p15-specific siRNA effectively silenced p15 expression, but up-regulated the p-gp expression in the HepG2/CDDP/2.0 cells. Apparently, treatment with the p15-specific siRNA might not increase the sensitivity of cells to CDDP, suggesting that combination with other strategies, such as CDKI or other chemotherapeutic reagents targeting multiple points of cell cycle, may be more effective in rescuing the sensitivity of tumor cells to chemotherapy. In summary, our data indicated that knockdown of p15 expression by siRNA promoted cell cycling and increased CDDP-resistance, which was associated with up-regulated expression of P-gp in HepG2/CDDP/2.0 cells. Therefore, our findings demonstrated that p15 is a negative regulator of cell cycling, associated with the development of CDDP-resistance in HepG2 cells. Conceivably, p15 may serve as a therapeutic target in the treatment of hepatocellular carcinoma.

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DECLARATION OF INTEREST

The authors have no conflict of interest to declare.

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